UNIVERSITA' DEGLI STUDI DI NAPOLI
FEDERICO II

PhD in

MODEL ORGANISMS
IN BIOMEDICAL AND VETERINARY
RESEARCH

XXIV CICLO
a.a. 2008/2009-2010/2011

PhD THESIS

ANATOMIC BRAIN ATLAS AND BRAIN MAP OF NEUROTROPHINS EXPRESSION IN A NEW MODEL ORGANISM: *Nothobranchius furzeri*

Advisor
Prof. Paolo de GIROLAMO

Author
Dr. Livia D'ANGELO

CONTENTS

TABLE OF CONTENTS

«Studia prima la scienza, e poi seguita la pratica, nata da essa scienza. Quelli che s'innamoran di pratica senza scienza son come 'l nocchier ch'entra in navilio senza timone o bussola, che mai ha certezza dove si vada»

«First study the science, then the practice born from this science. Those who fall in love with practice without science are like a sailor who enters a ship without a helm or a compass, and who never can be certain whither he is going»

Leonardo da Vinci

1. INTRODUCTION

1.1 *Neurotrophins*

Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin 4 (NT-4) and Neurotrophins 6 (NT-6) belong to the neurotrophin family. Historically, the first family member identified was NGF (Levi-Montalcini *et al.*, 1954). It was discovered during experiments of transplanting mouse sarcoma tissue into a chick embryo. The transplanted tumor tissue induced hyperinnervation of internal chick organs, by releasing a diffusible agent that stimulated the growth and differentiation of developing nerve cells. The discovery has lead, in the following years, to formulate the "neurotrophic hypothesis" (Purves, 1988). This theory is based on the assumption that the survival of neurons depends on the supply of a neurotrophic factor, synthesized in limiting amounts in the target fields. However, the neurotrophic hypothesis has been broadened by the demonstration that multiple neurotrophic factors regulate the survival of certain populations of neurons. Since the discovery of NGF, studies and findings have been continued, leading to exploration of the field of neurotrophins and identification of the other family members . Thus, in 1982 BDNF was purified from pig brain, thanks to its survival-promoting action on a subpopulation of dorsal root ganglion neurons (Barde *et al.*, 1982). Despite the different origin and distinct neuronal specificity, BDNF turned out to be a close relative of NGF. Few years later, the third member of the neurotrophin family, NT-3, was identified (Hohn *et al.*, 1990). NT-4 was isolated from Xenopus and viper (Hallböök *et a1*., 1991) first, and then, identified in both human and rat genomic DNA (Ip *et al.*, 1992), which represented the mammalian counterpart of Xenopus/viper NT-4. A fifth neurotrophin was then cloned from the human and named NT-5 (Berkemeier *et al.*, 1991; 1992). However, its chromosomal location and activity profile indicated that NT-5 was the ortholog of NT-4 (Berkemeier *et al.*, 1992).

Finally, NT-6 was only cloned from *Xiphophorus maculatus*, a teleost species (Götz *et al.*, 1994), and is not expressed in other vertebrates. Later findings had suggested the existence of an additional fish neurotrophin, NT-7, cloned in the

carp (Lai *et al.*, 1998) and in zebrafish (Nilsson *et al.*, 1998). However, Dethleffsen and colleagues (2003) provided strong evidence that NT-6 and NT-7, which had originally been considered different genes, have evolved from a single ancestral gene, which was generated by a single duplication of an ancestral NGF gene.

The interest in neurotrophins has never decreased over the years: beside the neurotrophic factor hypothesis, which continues to guide research on the effects of neurotrophic factors, further studies have shed light on other mechanisms of neurotrophic factor action, such as anterograde transport and presynaptic release of neurotrophins. The neurotrophins exhibit actions on distinct, as well as partially overlapping, subsets of peripheral and central neurons (for a review see Eide *et al.*, 1993; Ibáñez, 1995). Individual neurons may also be responsive to more than one neurotrophin at a given time, or at subsequent times during development. Moreover, the actions of neurotrophic factors are not restricted to developing neurons. Indeed, these factors can act on dividing neuroblasts as well as mature neurons that have established stable synaptic contacts. Thus, neurotrophins have profound influences, ranging from development, to differentiation, maintenance and plasticity of brain function throughout life. Loses in neurotrophins regulation and function place the nervous system at risk for cognitive malfunction and degeneration.

1.1.1 Biochemical features

All neurotrophins are structurally related proteins, whose genes code glycosylated neurotrophin precursors that are cleaved by pro-convertases to give rise to mature processed neurotrophins. Some features are conserved among neurotrophins such as 1) a presumptive signal peptide following the initiation codon (Leibrock *et al.*, 1989; Ernfors *et al.*, 1990; Hohn *et al.*, 1990; Jones and Reichardt, 1990; Kaisho *et al.*, 1990; Rosenthal *et al.*, 1990; Maisonpierre *et al.*, 1990, 1991; Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991; Ip *et al.*, 1992); 2) a pro-region, including an N-linked glycosylation site, and a proteolytic cleavage site for furin-like proteases followed by the mature sequence (Bresnahan *et al.*, 1990; Seidah *et al.*, 1996a, 1996b); 3) a distinctive three-dimensional structure determined by two pairs of anti-parallel b-strands, and six cysteine residues

forming three disulfide bridges, referred to as the cystine knot motif (McDonald and Hendrickson, 1993; Sun and Davies, 1995; Ibanez, 1998).

More in details, NGF consists of three subunits, a, b and g which interact to form a 7S complex of approximately 27 kDa in weight. The 7S complex contains two identical 118 amino acid b chains, which are solely responsible for the trophic activity of NGF. Mature b-NGF is synthesized from prepro-b NGF and exists within the CNS as a homodimer. While the b-NGF monomer also exhibits growth promoting activity, it is the dimeric form that is the principal and physiological mode (Maness *et al.*, 1994). BDNF is a basic dimeric 28-kDa protein of non-covalently linked 14-kDa subunits structurally related to NGF. Pro-BDNF, a 32-kDa precursor, undergoes cleavage to release mature 14-kDa BDNF protein as well as a minor truncated form of the precursor (28 kDa). BDNF gene consist of eight 5' untranslated exons and one protein coding 3' exon. Transcription of the gene results in BDNF transcripts containing one of the eight 5' exons spliced to the protein coding exon and in a transcript containing only 5' extended protein coding exon (Aid *et al.*, 2007). NT-3 is a polypeptide of 199 amino acids, and the mature rat NT-3 peptide displays a 57% amino acid identity with rat NGF and a 58% amino acid identity with rat BDNF. However, despite this structural homology, NT-3 has a distinct biological activity and a different spatiotemporal characteristic from both NGF and BDNF (Robinson *et al.*, 1995). NT-4 precursor protein is made of 236 amino acids, it is processed into a 123 amino acid mature NT-4 form (Hallböök *et al.*, 1991; Hallböök *et al.*, 1999). The structure of NT-6 precursor is formed of 286 aminoacids. NT-6 contains an additional basic domain of 22 amino acid residues between cysteine residues 2 and 3. The corresponding region in NGF forms a loop on the protein surface (Götz *et al.*, 1994). This domain might be responsible for the observed interaction of NT-6 with the glycosaminoglycan heparin that affects the release of NT-6 from the extracellular matrix and cell surface (Götz *et al.*, 1994). The mature part of all neurotrophins shows a high degree of sequence similarity, and approximately 50% of the amino acids are common to all neurotrophins. The mature proteins often build noncovalently linked homodimers as active forms (for review see Bibel and Barde, 2000).

1.1.2 Neurotrophins and receptors

The signal transduction systems, that mediate the diverse biological functions of the neurotrophins, are initiated through interactions with two categories of cell surface receptors, the p75 neurotrophin receptor and the tropomyosin-related kinase (Trk) tyrosine kinase receptors (for a review see Schecterson and Bothwell, 2010). p75 was the first receptor to be discovered and was identified as a low-affinity receptor for NGF, but was subsequently shown to bind each of the neurotrophins with a similar affinity (Rodriguez-Tebar *et al.,* 1991; Frade and Barde, 1998). p75 is a member of the tumour necrosis receptor superfamily with an extracellular domain that includes four cysteine-rich motifs, a single transmembrane domain and a cytoplasmic domain that includes a 'death' domain similar to those present in other members of this family (Liepinsh *et al.*,1997; He and Garcia, 2004). p75 binds to all members of the neurotrophin family, and even the proneurotrophins, along the interface between the two monomers and triggers a number of pathways depending on the cellular context. Among these, some pathways may indirectly end up in cell cycle regulation, such as activation of ceramide (Cer), c-Jun N-terminal kinase (JNK), Rac, NF-kB, and Ras. The other group of neurotrophins receptors is made of the subfamily of receptor tyrosine kinases (Trk) (Mitra *et al.,* 1987; Martin-Zanca *et al.,* 1989). Three distinct genes encoding Tks have been studied in vertebrates: TrkA, TrkB and TrkC (for a review see Huang and Reichardt, 2003). These are transmembrane receptors, with an extracellular domain which consists of a cysteine-rich cluster followed by three leucine-rich repeats and a cytoplasmic tyrosine kinase domain. Each receptor spans the membrane once and is terminated with a cytoplasmic domain consisting of a tyrosine kinase domain surrounded by several tyrosines that serve as phosphorylation- dependent docking sites for cytoplasmic adaptors and enzymes (Reichardt, 2006). Thus, the main mechanism of action, when Trks bind to neurotrophins, is the activation through transphosphorylation of the kinases present in their cytoplasmic domains. A number of intracellular signaling cascades including, among others, the ras/extracellular regulated kinase (erk) and the phosphatidylinositol-3 kinase (PI 3 kinase) cascades, which result in regulating the differentiation and survival of neurons, are activated. The binding to the receptors Trks or p75 is related to

different degree of affinity, depending also on different binding sites. Affinity to the specific neurotrophin determines both the responsiveness and specificity of neurotrophin itself. Trk receptors show high binding affinity: NGF and NT-6 binds preferentially to TrkA; BDNF and NT-4 to TrkB; and NT-3 to TrkC.

Further studies have highlighted that the binding of NGF to TrkA, and BDNF to TrkB is of low affinity but it can be regulated by receptor dimerization, structural modifications or association with the p75 receptor (Arevalo *et al.*, 2000; Esposito *et al.*, 2001). The p75 receptor can bind to each neurotrophin, and also acts as co-receptor for Trk receptors as shown in Fig 1. Expression of p75 can increase the affinity of TrkA for NGF and can enhance its specificity for cognate neurotrophins (Hempstead *et al.*, 1991; Benedetti *et al.*, 1993; Bibel *et al.*, 1999). As a result, increased ligand selectivity can be conferred on the Trk receptors by the p75 receptor. In addition to forming complexes, Trk and p75 receptors show independent signalling properties, and downstream signal transduction pathways significantly contribute to individual physiological responses.

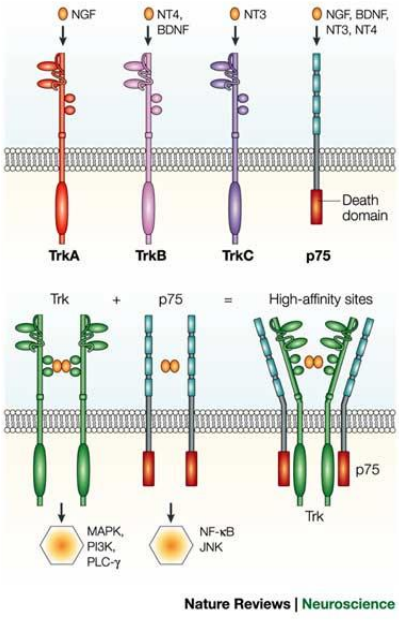


Fig 1. Neurotrophins bind selectively to specific Trk receptors, whereas all neurotrophins bind to p75. Neurotrophin binding to the p75 receptor mediates survival, cell migration and myelination through several signalling pathways. Interactions between Trk and p75 receptors can lead to changes in the binding affinity for neurotrophins. **Abbreviations:** JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NT, neurotrophin; PI3K, phosphatidylinositol 3-kinase; PLC-γ, phospholipase Cγ. (Chao, 2003)

The biological effects of neurotrophins require that signals be conveyed over long distances. After neurotrophin-mediated activation, the receptors shuttle back from the axon terminals to the soma by retrograde transport in so-called signaling endosomes to regulate gene expression and exert their trophic responses. Endosomes are delivered to the cell body where signals are transduced to ensure neuronal survival and differentiation. Endosomes serve as a meeting point for the formation of signaling complexes for a wide variety of ligands and their receptors. Since the formation of transport vesicles would orient the intracellular domain of the receptors to face the cytoplasm, each receptor would be available for interactions with cytoplasmic proteins and produce many signaling possibilities (for a review see Chao, 2003). The retrograde trafficking process is well studied, and is becoming increasingly clear (Hirokawa *et al.*, 2009). Conversely, assumed mechanisms underlying anterograde Trk transport remain incompletely understood. Possible explanations on anterograde axonal transport involves heatshock proteins, such as HSP90, which bind to TrkB during axonal transport (Bernstein *et al.*, 2001), and APP, which may be a kinesin endosome receptor for a subset of transport vesicles (Kamal *et al.*, 2000). Because multiple kinesin isoforms are expressed in neurons, it could be hypothesized that different kinesins may be responsible for anterograde axonal transport of different types of endosomes, possibly facilitating their targeting to different destinations or uses, but as of yet, there is little direct evidence for this mechanism. However, recent evidences have indicated the involvement of sortilin as an important anterograde trafficking receptor for the Trk proteins (Vaegter *et al.*, 2010).

1.1.3 Biological activities

The neurotrophin proteins are crucially involved in regulating the survival and differentiation of neuronal populations during development (Levi-Montalcini, 1987; Davies, 1994; Lewin and Barde, 1996; Chao, 2003). In addition to these well-established functions in development, a large body of work suggests that neurotrophins continue to shape neuronal structure and function throughout life (Schnell *et al.*, 1994; Thoenen, 1995; Bonhoeffer, 1996; Prakash *et al.*, 1996).

For example, NGF has been shown to influence neuronal soma size, dendritic complexity **(**Ruit *et a1*., 1990), and the number and size of synaptic contacts in the brain (Garofalo *et al.*, 1992). Other neurotrophins may have similar effects on different central nervous system populations, and long-term morphological changes are of obvious importance in functional connectivity (Lisman and Harris, 1993; Pierce and Lewin, 1994). Neurotrophins such as BDNF and NT-3 produce rapid increases in synaptic strength in nerve–muscle synapses, as well as increases in excitatory postsynaptic currents in hippocampal neurons (Jacobi *et al.*, 2010). BDNF and NT-3 also induce rapid and long-lasting enhancement of synaptic strength through LTP in hippocampal cells cultures.

Additionally, neurotrophins represent strong candidates in regulating the continued plasticity of multiple brain systems long beyond the early organization that occurs during embryonic and perinatal period and adulthood. This is particularly true for BDNF, a key molecule critical in the control of neurogenesis, since it impacts cell proliferation within the neurogenic zone of mammalian brain (Bath and Lee, 2010), and contributes to the process of neuroblast migration (for a review see Bath and Lee, 2010). Well established is also the role of neurotrophins in the regeneration processes. In fact, it has been noted that neurotrophin secretion increases in the nervous system following injury. BDNF and NGF secretion increases dramatically within hippocampal neurons depolarized by kainic acid (Zafra *et al.*, 1990) or following ischemic injury (Lindvall *et al.*, 1992). Although Schwann cells, fibroblasts, and activated mast cells normally synthesize NGF constitutively, direct trauma and induced cytokines combine to increase neurotrophin production in these cells after injury (for a review see Levi-Montalcini *et al.*, 1996). Among all neurotrophins, the actions of BDNF on central neurons have been best characterized. Indeed, modest changes in BDNF levels or expression of altered single nucleotide polymorphism in the BDNF gene is correlated with neuroanatomical differences and behavioral changes in humans and in mouse models, including abnormal feeding behavior, alterations in episodic memory, and susceptibility to neuropsychiatric disorders of anxiety and depression (Lyons *et al.*, 1999; Egan *et al.*, 2003; Chen *et al.*, 2006; Castren and Rantamaki, 2010; Russo *et al.*, 2009).

However, changing in the regulation and function of all members of neurotrophin family place the nervous system at risk for cognitive malfunction and degeneration. In the brain aging and in neurodegenerative disorders, there is a fragile balance between neurotrophic factor support and dysfunction. Many evidences highlight the strong implication of neurotorphic factors and neurotrophins in particular in CNS disfunction (for a review see Connor and Dragunow, 1998). It is unclear whether alterations in neurotrophins and their receptors expression are primary or secondary to the neuropathological changes associated with each disorder. Indeed, while it has not been determined whether those alterations precede the development of the disease process, it is possible that the observed alterations in neurotrophins or receptors may be involved in the primary pathogenesis of specific neurodegenerative disorders (for a review see Connor and Dragunow, 1998). Based on these observations, members of the neurotrophins family could have potential therapeutic roles in preventing and, or reducing the neuronal cell loss and atrophy that occurs in neuronal aging and in neurodegenerative disorders such as Alzheimer diseases, Parkinson's disease, Huntington's disease or amyotrophic lateral sclerosis (ALS).

1.1.4 Neurotrophins as Therapeutic Agents

The involvement of neurotrophins in biological processes linked to the emergence of severe and widespread pathologies has suggested their use as promising therapeutic agents. Several investigations have provided support for this idea (Covaceuszach *et al.*, 2009), however, the use of neurotrophins as drugs has been hampered by their poor pharmaceutical properties (Stanzione *et al.*, 2010). Clinical trials using neurotrophins molecules have been performed in some neurodegenerative diseases, such as Alzheimer disease, Parkinson disease, Hungtinton disease, ALS (for a review see Hefti, 1997).

Basically, two approaches are currently studied. First, the development of mimetics endowed with neurotrophin-like activities: neurotrophin proteins could be replaced by active peptide fragments or molecules that mimic the active sites of neurotrophic factors. Second, the specific manipulation at any step in the synthesis, release, and transduction mechanism of endogenous growth factors. The neuroprotective effects of BDNF in rodent and primate of Alzheimer's

disease demonstrated efficacy of intraventricular infusions of growth factors and intraparenchymal infusions or gene delivery (Nagahara *et al.*, 2009). The broad neuroprotective effects of entorhinal BDNF administration in several animal models of Alzheimer's disease, with extension of therapeutic benefits into the degenerating hippocampus of rodents and primates, provides a rationale for exploring clinical translation (Nagahara *et al.*, 2009).

1.1.5 Phylogenesis of neurotrophins

Neurotrophins and Trk receptors represent good examples of molecules driving experience-dependent changes involving neuronal selection, survival and plasticity within the nervous system. Thus, genes that drive such experience-dependent changes are subject to selection. Indeed, genes encoding neurotrophins molecules have undergone molecular changes during evolution.

All neurotrophins contain a highly conserved C-terminal domain and bind to the same receptor family. Both correct folding and post-translational processing of the entire pre-proprotein are necessary for sorting to the extracellular space, dimerization and receptor binding. Studies of molecular evolution have highlighted that neurotrophins are highly conserved in vertebrates evolution. Neurotrophins and their cognate Trk receptors have been found in vertebrates including reptiles, amphibians, fish, birds and mammals (for a review see Hallböök *et al.*, 2006), while NT-4 has not been found in chicken, the only bird species whose complete genome is currently available (Hallböök *et al.*, 1991), and NT-6 has been isolated only in fish (Götz *et al.*, 1994). A phylogenetic analysis of the neurotrophin and Trk gene families has been performed either on mature aminoacid sequences and on nucleotide sequences of selected organisms at different evolutionary stages (Hallböök *et al.*, 1991; van Kesteren *et al.*, 1998; Hallböök *et al.*, 1999; Lanave *et al.*, 2007). The analysis supports the notion that neurotrophins evolved early in vertebrate history during the two rounds of genome duplication that characterized the origin and evolution of vertebrates (Hallböök *et al.*, 2006). This evidence has been further confirmed in the detailed analysis of the study on BDNF phylogenesis in vertebrates (Tettamanti *et al.*, 2010), where the coding sequence for the BDNF protein in 36 different vertebrates, ranging from mammals to fish, was investigated to evaluate

whether the evolutionary selection played a major role in the evolution of the neurotrophins (Tettamanti *et al.*, 2010). BDNF cDNA underwent some degree of selective pressure in molecular evolution through the vertebrate phylogenesis, clustering together species belonging to the same class. An adaptive type of selective pressure seemed to be responsible for clustering of mammals apart from other classes. The mutants arose from point mutations as well as insertion/deletions modalities: these last are especially represented in teleosts. Differently conserved regions are recognized along the sequences, corresponding to different parts of the transcript. In fish, the knowledge of neurotrophins is rather limited. Only recently, several researches, supported by phylogenetic analysis, have turned the attention on the biological role of neurotrophins by using fish as alternative model to rodents. Significant advances have been made in the field of neurobiology of fish and neurotrophins occupy a rather large space.

1.1.6 Neurotrophins in fish

In fish, synthesis and activity of neurotrophins have been studied (for a review see Heinrich and Lum, 2000), and investigations have been mainly devoted to characterize the molecular aspects of neurotrophins. In fish, as in mammals, neurotrophins are synthesized as precursors, and then they are cleaved to get the mature form.

Information on the primary structure of all fish neurotrophins have been gathered from cloned cDNAs or genes, following cloning and nucleotide sequencing (Heinrich and Lum, 2000). The overall organization of all neurotrophin genes is similar: there is one major exon, enconding the whole precursor. Slight differences can be noticed among all neurotrophins. BDNF-encoding exon possesses four upstream exons and a separate promoter. The mature BDNF transcripts possess two exons: the first is one of the four upstream exons and the second is the BDNF-encoding major exon (Hashimoto and Heinrich, 1997). NT-3 gene has a similar organization, characterized by two upstream exons possessing a separate promoter (Leingartner and Lindholm, 1994).

NGF transcripts contain up to four exons encoding a NGF precursor that is extended at the N-terminus. The upstream organization of the NT-4 gene is similar to that of the NT-3 gene. There are two promoters, each generating a different 5' end of the NT-3 transcripts. All transcripts therefore contain two exons. The second exon encodes the NT-4 precursor (Heinrich and Lum, 2000). The organization of the NT-6 presents 4 exons (Dethleffsen *et al.,* 2003). With regards to the structures of proteins, fish neurotrophins have the same three-dimensional structure as the mammalian ones. Studies based on crystallography and mutagenesis have revealed that the amino acid residues have three-dimensional structure (Götz *et al.*, 1994). Moreover, like in mammals, neurotrophins are able to form dimers, since they activate the autophosphorylation of Trk receptors and to do that, they require dimers (Woo *et al.*, 1998).

Neurotrophin genes are expressed in fish at the RNA level (Ip *et al.,* 1992; Hashimoto and Heinrich, 1997), both in development and adulthood.

The functional roles of neurotrophins in fish is still matter of debate. So far, hypothesis have been formulated. Actions may correlated to regulation of programmed neuron death and survival during the development and in the adulthood (Eisen *et al.,* 1990; Pike *et al.,* 1992). Furthermore, neurotrophins can be deeply involved in the regeneration and neurogenic activities of fish. Teleost fish possess the considerable capacity to generate new neurons in the adult CNS, the so-called “adult neurogenesis”, and to regenerate whole neurons after injury, the “neuronal regeneration”, in contrast to limited plasticity of the adult mammalian CNS (Zupanc and Sîrbulescu, 2011). Neurotrophins, and particularly BDNF, may represent a strong candidate in regulating the continued plasticity of multiple brain systems long beyond the early organization that occurs during embryonic and perinatal period (Bath *et al.*, 2011).

1.2 *Nothobranchius furzeri*

Fig 2. *N. furzeri.* Strain MZM8 (long-lived phenotype). Adult male.

1.2.1 *Nothobranchius furzeri*

Nothobranchius furzeri is a Teleost fish, member of the superorder Atherinomorpha, of the order Cyprinodontiformes.

The name *Nothobranchius* is due to a peculiarity: the presence of a fake gill opening in the operculum, *furzeri* originates from the name of the collector Furzer, who collected eggs in South-Eastern Africa over 40 years ago. Indeed, *Nothobranchius furzeri* is an African Annual fish, which origins mainly from Mozambique and Zimbabwe. This Annual fish inhabit ephemeral pools (typically on flood plains) in Eastern Africa and have adapted to the routine drying of their environment by evolving desiccation resistant eggs that can remain dormant in the mud for one and maybe more years (Genade *et al.*, 2005). The life expectancy in the wilderness cannot exceed the seasonal duration of the habitat, which is in the order of months. In captivity, the life span is likewise short even in constant presence of water.

To date, different strains of *N. furzeri* have been collected and are distinguished by the collection places. The strain originating from Mozambique is characterized by long-lived phenotype (median life-span is up to 12 months) while the strain from Zimbabwe comprises extremely short-lived phenotype (median life-span is of just three months) (Terzibasi *et al.*, 2007).

These animals are extremely sexually dimorphic and dichromatic; males are robust and colourful, and females are smaller and pale yellow or brown. Females of related species can hardly be distinguished morphologically and the classification is based mainly on male livery and size. They are relatively small fishes with a median size of 5 cm (ranging from 3 to 15 cm). These fishes are all adapted to survive in temporary waters and all produce eggs which not only resist desiccation but require a dry period to develop correctly, at least in captivity. They all express a stereotyped behavioural pattern in which the male drives the female toward the substrate, in order to lay eggs in the muddy bottom.

1.2.2 Phylogeny

The order Cyprinodontiformes comprises *Fundulus heteroclitus* (the so-called mumi-chog), *Poecilia reticulata* (the popular aquarium fish guppy) and *Xiphophorus* (the platyfish).

Molecular phylogenetic analyses, based on whole mitogenome sequences (Setiamarga *et al*., 2008), have provided evidence that the order Cyprinodontiformes is a sister group of Beloniformes, whose main representant is *Orizyas latipes*, well known as medaka.

All these species are widely employed in biomedical research for studies of ecology, evolution and genomic resources. Therefore, taking into account the phylogenetic analyses, *Nothobranchius* occupies a very convenient position for performing comparative genomics and molecular studies.

The genus *Nothobranchius* includes about 50 currently described species, separated into 5 well-defined clades (Huber, 2005; Wildekamp, 2004) and a series of other taxa. The different species present a biodiversity, accoding to the variations of regions where they come from.

Remarkable variations in regional climate and precipitation patterns are observed in *Nothobranchius* habitats. Some (e.g. Zanzibar) have two short dry seasons which punctuate the long wet season (Wildekamp 2004), while other localities may not dry out at all in some years (e.g. Mafia Island) (Seegers, 1997). By contrast, some of the locations where *Nothobranchius* is found, such as Sudan or the Kruger National Park, show brief and erratic rainy seasons and may not receive sufficient rain for several years (Jubb, 1981).

Differences in precipitation patterns result in large-scale differences in captive longevity not only for different *Nothobranchius* species, but also for different populations of the same species.

1.2.3 Distribution, polymorphism and habitat

Data on the distribution, ecology and demography of *N. furzeri* are essential for their full appreciation in studies investigating the evolutionary origin and consequences of ageing (Genade *et al.*, 2005). The distribution range is between Southern Mozambique and Gona Re Zhou Game Reserve in Zimbabwe. More precisely, the distribution range comprises three broadly-defined watersheds. The first is the set of temporary rivers that flow from the plateau of Gona Re Zhou (about 400m) in Zimbabwe into Mozambique. This watershed is referred to as the "Chefu" basin. The second watershed is the Limpopo. The third watershed is defined by the rivers Vaneteze and Mazimechopes and draws into the Incomati. The river Save represents the western limit of the distribution range for *N. furzeri*.

According to different distribution, two colour morphs are expressed in male of *N. furzeri*. In the type population in the upper Chefu basin, only yellow males were found (Jubbs, 1972). In the lower Chefu basin, all populations were composed of a mixture of yellow and red males, while only 7 out of 13 populations in the Limpopo basin contained any yellow males. The yellow morph also dominated in the Incomati basin in the south-western part of the *N. furzeri* range, with exclusively yellow males found in the Vaneteze basin and yellow-dominated populations in the Mazimechopes basin. In contrast, the red morph dominated in most populations in the Limpopo basin that includes the centre and the largest part of the *N. furzeri* range (Reichard *et al*., 2009).

The habitat of *Nothobranchius* is characterized by vertisol soils on alluvial deposits (Wildekamp, 2004), because vertisols provide suitable soil structure for survival of dormant eggs during the dry season (Wildekamp, 2004). *N. furzeri* often inhabited simple shallow pools without any vegetation. Within pools with vegetation, *N. furzeri* typically occurred in *Nymphaea* in close proximity to open water or at the interface between vegetation and open water.

1.2.4 Life cycle

N. furzeri is an Annual fish. As the name suggests, the life cycle is completed within a year: the eggs require a dry period to develop fully. As previously described, these fish inhabit areas that are subject to seasonal rainfall - creating bodies of water. As the year progresses, the water levels drop, and after several months dry up completely. They bury their eggs in the substrate, and die when the pool evapourates. A few months later, the rains come again, bringing life to the pool, and allowing the now fully developed embryos to hatch. The weather is always unpredictable, so the fry must grow quickly, reach maturity and reproduce before the pool dries up again. In general they are fast growers, reaching adulthood after a few weeks. Sometimes, the fish do not reach the adult stage, however, this does not spell the end for the population. The eggs go through several distinct stages in their growth, including resting periods called diapauses.

Under laboratory conditions, diapause is characterized by three stages (Wourms 1972a; Wourms 1972b; Wourms 1972c). In DI (the first diapause) blastogenesis has completed and embryogenesis has not yet begun. At DII embryogenesis is complete and organogenesis has not begun. At DIII organogenesis is complete and the fully formed fry awaits the environmental cue to hatch.

1.2.5 *Nothobranchius furzeri* as model organism

In biomedical and veterinary research, vertebrate and invertebrate model organisms are widely employed: they present great advantages for identifying and understanding genes that are altered in heritable human diseases. The functions of many of those genes, have remained largely intact in both vertebrate and invertebrate lineages during the ensuing course of evolution. Among vertebrates, fish occupy a special position: their organ systems, including the brain, the heart, the hematopoietic system, and the digestive and excretory organs, are similar to those of other vertebrates. Fish are useful model for ageing research: their short lifespans, the relatively low cost of maintaining large numbers of individuals for lifetime studies, and in many cases, a well-developed genetics. Particularly, *N. furzeri* has been proposed as

a new model system for aging research (Genade *et al.*, 2005; Terzibasi *et al.*, 2007): it is the shortest-lived vertebrate which can be cultured in captivity (Valdesalici and Cellerino, 2003). *N. furzeri* can be cultured, and their eggs stored dry at room temperature for months or years, offering inexpensive methods of embryo storage; they show accelerated growth and expression of aging biomarkers at the level of histology and behaviour; the so-short lifespan offers the possibility to perform investigations thus far unthinkable in a vertebrate, such as drug screening with life-long pharmacological treatments and experimental evolution; when the lifespan of different species is compared, a general correlation is found between wet season duration in their natural habitat and longevity in captivity; and vertebrate aging-related genes, such as *p66Shc* and *MTP* can be easily isolated in *Nothobranchius* by homology cloning.

1.3 Scientific significance of teleost fish for neurobiological research

The brain of Teleosts has gained considerable importance in the neuroscience field: the use of “neurobiological models”, that are “simpler” than the mammalian brain, might facilitate research into central nervous system (CNS). Studies in the basic neurosciences have been focused predominantly upon rat and mouse models. The majority of studies conducted are performed on either rats or mice, with humans as a close second. A confirmation of this status comes from an analysis of Manger and coworkers (2008), who analysed publications in the basic neurosciences (to the exclusion of the clinical neurosciences) from 12 general neuroscience journals covering a variety of specializations for the years 2000–2004, plus two journals that specifically target the comparative neuroscience community (*Brain, Behavior and Evolution* and *The Journal of Comparative Neurology*) over the period 1975–2004. What they assessed was that studies on species other than rodents are more limited, and the proportion of journal space dedicated to the various species that form the objects of scientific study is rather low.

However, the complete genomes of many species, the accelerating pace of bioinformatics as a directive research tool, and the technical advances being made especially in molecular and developmental neuroscience support and reinforce the study of species with phenotypes different to rats and mice, such as fish. Comparative neurobiology can provide insight about the human nervous system and its role in health and pathological conditions. Fish models can lead to understanding of vertebrate neurology in general, and provide perspective by their fundamental evolutionary relationship with other vertebrates. Certain progressive neurological diseases are best understood in the context of the evolutionary states of the nervous system in which evolutionarily "higher" CNS functions are lost first, then sequentially "lower" evolutionary states, with the order being reversed during recovery.

The main architecture of the fish brain resembles that of the mammals, despite differences mainly in the development of the telencephalon. Although they

cannot replace clinical research, there is much to be learnt from fish brains. The knowledge of the brain architecture of fish is necessary to study the neural basis of behavior and mechanisms relevant for neurological processes and neurodegenerative diseases.

In fish, as in other vertebrates, two prominent external constrictions divide the early developing brain (neural tube) into the three neural vesicles giving rise to the basic regions of the adult brain: forebrain (or prosencephalon), midbrain (or mesencephalon), and hindbrain (or rhombencephalon). These vesicles represent the fundamental antero-posterior (i.e., rostrocaudal) subdivisions that later on may become subdivided into more or less evident transverse bands or neuromeres, which have defined relations with other structures of the head and the peripheral nervous system, being very useful in comparative studies. Because the developmental genes that are involved in mammalian brain development are largely conserved, comparisons between fish and mammalian brain development can be made through the analysis of gene expression.

The analysis of gene and the anatomical studies combined with the use of molecular techniques could lead to significant advances in the understanding of human neural disorders as well as human brain evolution.

1.3.1 The central nervous system of teleosts

The teleost brain is composed of three major regions: the forebrain, midbrain, and rhombencephalon. The forebrain can be further subdivided into the telencephalon and diencephalon. The midbrain is subdivided into a dorsal part, entirely occupied by lobes of optic tectum, and the ventrally located tegmentum. The rhombencephalon encompasses cerebellum and medulla oblongata, respectively dorsal and ventral positioned, and does possess structures that are present in the mammalian pontine tegmentum (the tegmental region dorsal to the pons *sensu stricto*). The above-mentioned structures can be identified in teleosts as well, e.g. the locus coeruleus and the principal trigeminal nucleus, and this region may be called the “pontine area.” In teleosts, the latter area includes the lateral nucleus of valvula which receives telencephalic inputs and projects fibers to the cerebellum. The nucleus may

share a similar ontogenetic origin to the mammalian pontine nuclei, suggesting their homology (Yang *et al.*, 2004).

Telencephalon

The telencephalon represents the most rostral part of encephalon. It consists mainly of olphactory bulbs and telencephalon sensu strictu or telencephalic hemispheres. The olphactory bulbs are sessile and organized in layers, whose boundaries are not very distinct. From the most external layer, it can be recognized: an outer layer of primary olphactory afferent fibers, a glomerular layer, a layer of mitral cells or external cellular layer and an inner layer of granule cells. The olphactory bulbs receive a so-called primary olphactory input from sensory nerve cells in the sensory epithelium of the olphactory organ. The pathway is via the olphactory nerve. Both the bulbs are interconnected via the medial oplhactory tract and project predominantly to the ventral area of telencephalon and dorso-posterior telencephalon. The telencephalon is subdivided into a dorsal and ventral area, each with a number of cytoarchitectonically delineable subdivisions. In the dorsal area, it is possible to recognize dorso-lateral, dorso-dorsal, dorso-medial and dorso-central areas. Dorso-lateral and dorso-medial areas present a high density of small neurons, mainly organized in layers, whereas the dorso-central area consists of larger neurons. The dorso-posterior telencephalon is an important source of olphactory bulbs afferents and consists mainly of fibers. The ventral area is divided into a ventro-dorsal, ventro-ventral, ventro-lateral areas, supracommissural and post-commissural parts. All these subdivisions are mainly characterized by small neurons and neuronal processes projecting back to olphactory bulbs or to midbrain regions.

Diencephalon

The diencephalon is located between the telencephalon and mesencephalon: the rostral boundary is the preoptic area and the caudal boundary is the pretectal region. The diencephalon is subdivided into epithalamus, thalamus, and hypothalamus zone. The preoptic area is regarded as the most rostral diencephalic subdivision, and includes the preoptic and suprachiasmatic nuclei.

By cytomorphological criteria, the preoptic nucleus is split into a magnocellular and parvocellular portions, respectively located dorsally and superiorly, in the periventricular zone. The epithalamus represents the rostrodorsal part of diencephalon and comprises the habenular nuclei, the epiphysis and the dorsal saccus. Habenular nuclei and epiphysis are interconnected structures, since input arise from one and reach the other. Moreover, epiphysis plays a key role in the synchronization of activity rythms with the dark-light cycle. The thalamic "complex" includes a dorsal and ventral thalamus. This complex is the major sensory relay station of the brain, receiving all inputs (except olfaction) and connecting reciprocally with the overlying structures. Particularly, thalamic nuclei are characterized as a multimodal relay centre, relaying visual, somatosensory, acoustico-lateral and cerebellar input to the telencephalon. The dorsal thalamus of teleosts does not appear to serve as the major sensory relay center to the telencephalon (Northcutt, 2006); whereas accumulating evidences suggest that the preglomerular complex, located ventrolateral in the thalamus, serves as the major sensory relay station to the telencephalon (Northcutt, 2006). The hypothalamus, as the name implies, is located below the thalamus and represents the ventralmost diencephalic region. It is connected with the hypophysis by means the pituitary stalk, which contains hypothalamic and preoptic nerve fibers involved in neuroendocrine regulatory functions. In the teleostean hypothalamus, a periventricular zone and inferior lobe can be recognized. Finally, the pretectal region is located in front of the rostral margin of the optic tectum and includes both diencephalic and mesencephalic structures. Functionally, the pretectum may be characterized as a primarily visual region, receiving both retinal and tectal visual input.

Mesencephalon

The mesencephalon or midbrain consists of a dorsally located tectum, named optic tectum, a ventromedially located tegmentum and a ventrolaterally located semicircular tori. This general subdivision reflects a diverse functional role of the structures. Indeed, the optic tectum is mainly involved in sensory functions, whereas the ventromedial and ventrolateral structures have predominantly

involved in (pre)motor functions. The optic tectum is a paired structure, organized in layers. Anteriorly, at the medial tectal margin there is a paired structure, named longitudinal tori, one of the main source of visual projection to optic tectum. The optic tectum is not only involved in visual inputs processing, but also in integration of visual signals with sensory information, and in generation and coordination of goal directed movements. Thus, it is considered as one of the main sensorimotor integration centers of the teleostean CNS. The tegmentum encompasses many motor structures, such as the oculomotor and trochlear nuclei, and continues into the medulla oblongata without any sharpen boundary.

Rhombencephalon

The rhombencephalon is the most caudal part of the brain. Caudally it continues gradually in the spinal cord. It is composed of the dorsoanterior cerebellum, the ventroanterior pons and posterior ventrally located medulla oblongata. The cerebellum basically consists of three main regions: the valvula, the part protruding rostrally into the mesencephalic ventricle, the corpus, centrally located, and the caudal cerebellar region which consists of the caudal lobe and granular eminentiae. The primary role of the cerebellum is to coordinate movement, and it thus receives information from many sources while transmitting information primarily to motor cortical areas.

The medulla forms the fourth ventricle of the brain, and together with the midbrain, are often referred to as the "brainstem". The brainstem contains a conglomerate of cell groups that form a complex network termed the reticular formation, which is involved in higher order behaviors such as respiration, circulation, and wakefulness. The hindbrain also contributes 8 of the 10 pairs of cranial nerves, numbers V through X. Cranial nerves can have both motor and sensory components, and those derived from the hindbrain are responsible for a wide variety of behaviors, including taste, hearing, balance, mastication, facial expressions, some eye movements, and, in terrestrial vertebrates, the secretion of tears and saliva.

2. AIMS

In biomedical and veterinary research, growing interests have been shifted to study alternative model organisms, which could replace the traditional ones. The sought model should be easy to manipulate and manage, economically advantageous, simple for anatomical and physiological organization. Particularly in aging and in neurobiology research, to explore the root causes of aging processes of brain, fish are useful models, because most of their organ systems, including the brain, the heart, the hematopoietic system, the digestive and excretory organs, are similar to those of other vertebrates.

The main core of the thesis is a neuroanatomical study of the CNS of the teleost model *N. furzeri,* whose biological features make it suitable for aging research (Terzibasi *et al.*, 2007).

The first step of the research project was the neuroanatomical study of the brain of *N. furzeri.* Atlases of other teleostean species are available, however given the high interspecies variability existing in fish, the neuroanatomical differences raised the need to realize a specific atlas of the CNS of *N. furzeri.* Therefore, the main structures and neural circuits of central nervous system of this teleost were identified and characterized and the schematic drawings are reported.

Then, the research has continued aiming at investigating the presence and localization of neurotrophins, specifically BDNF, NGF, NT-3, NT-4 and NT-6, in the adult CNS of the teleost *N. furzeri*. Neurotrophins are members of the growth factor family, which play crucial roles in both the developing and mature mammalian nervous system. They are important during development in regulating neuronal survival, promoting axonal pathfinding and elongation towards targets, and in supporting synaptic plasticity (Gillespie, 2003; Cohen-Cory and Lom, 2004), and are involved in neuronal plasticity and survival in the adult nervous system. Thus, physiologic or pathologic age-associated changes in the CNS might be attributable also to an impairment of regulation of neurotrophins in brain.

Previous studies in lower vertebrates have shed light on the role of neurotrophins during developmental stages or regenerative processes after trauma or injury of nervous system (Dalton *et al.*, 2009; Germanà *et al.*, 2010a,b; Laramore *et al.*,

2011), but no extensive studies have been performed on the whole brain of an adult teleost. Moreover, fish possess the ability to generate new neurons at all stages of life. This ability may involve novel functions of and responses to neurotrophins and likely mimes this ``natural act'' in the human brain. In order to gain insight, ISH was carried out on brain sections by testing a “classical” and a new type of probe. The expression gene pattern has been completed by the study of distribution of each neurotrophin protein, by employing immunohistochemistry technique. The detailed mapping of neurotrophins expression and distribution throughout the CNS of *N. furzeri* may help to characterize the functional mechanisms into different regions of the teleost brain.

3. MATERIALS AND METHODS

3.1 Animals

For this study, wild-type *N. furzeri* from the strain WZZM 07/10 were employed. 40 adult fish were housed in 40L tanks at 25°C, with an "air supplied" water filtration and low water flow. Food was supplied twice a day, and consisted of commercially available, frozen bloodworm larvae (*Chironomus sp*.). Fish were maintained in 12 hours light/dark cycles. Adult subjects were let spawning on a river sand substrate, which was weekly sieved and returned to the bottom of the tanks. For the experimental procedures, all animals were anesthetized with 0.1% ethyl 3-aminobenzoate, methanesulfonate (MS-222; Sigma-Aldrich Chemical Co., St. Louis, MO).

3.2 Solutions

Solutions were prepared with bidistillate water. Solutions were autoclaved for 20 min at 121 bar or filter sterilized (0.2-0.45 μm pore diameter). Glassware was autoclaved. For RNA-work, solutions and water were treated with 0.1% diethylpyrocarbonate (DEPC), shaked vigorously and mixed for about 20 min on a magnetic stirrer to bring the DEPC into solution. The solutions were then autoclaved to remove any trace of DEPC. Tris buffers cannot be treated with DEPC because it reacts with primary amines. DEPC decomposes rapidly into CO_2 and ethanol in the presence of Tris buffers. Therefore, Tris buffers were prepared by using water that has been treated with DEPC first. Standard stock solutions like EDTA, Tris, TAE, TBE, TE, PBS, SDS, SSC, NaOAc and culture media like LB and SOC were prepared as described in Table 1. All solutions used are named in the text.

3.3 Equipment

General lab equipment was used for the molecular and cell biology techniques, including balances, centrifuges, electrophoresis equipment, heating blocks and plates, hybridization and incubation ovens, micropipettes, PCR and gradient

thermocyclers, pH meter, shakers, sterile hood, UV-transilluminator, vortexes and waterbaths. Fresh frozen sections were obtained using the Cryostat CM 1900, Leica (Leica, Solms Germany).

Zeiss AxioScipe AX 1.0 microscope (Carl Zeiss, Jena, Germany) with AxioCam MC5 and AxioVision software was used to collect fluorescent microscopic images. Leica microscope DM RA2 (Leica Camera AG) attached to a Leica DC300 F camera for light microscopic images.

3.4 Molecular Biological Techniques

3.4.1 Isolation and Purification of DNA and RNA

a) Isolation of Genomic DNA

Genomic DNA was isolated from adults *N. furzeri.* The brains were rapidly excised from anesthetized animals and frozen in liquid nitrogen and pulverized. After addition of lysis buffer (0.1 M Tris/HCl, 0.2 M NaCl, 5 mM EDTA, 0.2% w/v SDS, pH 8.5) and proteinase K (150 µg/ml), the tissue was incubated under continuous rotation at 55°C overnight. Undissolved material was pelleted. After a phenol/chloroform extraction the DNA was precipitated using 1/10 volume of 3 M NaOAc (pH5.2) and 2 volumes of 100% ethanol. The DNA was washed two times with 70% ethanol, dried and dissolved in 100-500 µl H_2O at 4°C overnight.

b) Isolation of tissue specific RNA

Total RNA was extracted from the brains of adult *N. furzeri* using INVITROGEN Trizol following the manufacturer instructions. The tissues were centrifuged and homogenized at 4°C per 10 min at 12000 rpm. RNA, present in the aqueous phase, was transferred in a 1.5 ml sterile tube and precipitation was carried out by adding 0.5 ml isopropyl alcohol and centrifuging the sample at 4°C per 15 min at 12000 rpm. The pellet, containing the RNA, was resuspended in 100 µl of RNAse-free H_2O and stored at -80°C.

3.4.2 Polymerase Chain Reactions (PCR)

Polymerase chain reactions were performed for different purposes like subcloning of DNA fragments, identification of positive clones and determination of insert sizes in colony PCRs, preparing of a template for in vitro transcription and sequencing. Primers were designed according to standard methods using the Primer3 software. Different cycles conditions were used, according to the different purposes.

3.4.3 Oligonucleotide Primers

Oligonucleotide primers were purchased from MWG. The primers were delivered or dissolved at standard concentration of 100 mM. Working dilutions were prepared at concentration of 10 mM and stored at -20°C. All used are listed below: sequence(5'_3') T (°C) Supplier Purpose :

M13-Fwd GTAAAACGACGGCCAGT,

M13-Rev AACAGCTATGACCATG,

BDNF Fw ATGACCATCCTGTTC(C/G=S)TTACTA

BDNF Rev AAGACGGC(G/C/A=V)ATAGACATGTC

3.4.4 RT- PCR (first-strand reaction)

The isolated and purified RNA was used to synthesize the first filament of cDNA by using the kit SuperScript™ II Reverse Transcriptase (Invitrogen Life Technologies; New York, USA), according to the manufacturer instructions. The reaction mixture was prepared using 8 µl DNAse-treated RNA, 15 µl DEPC treated H_2O and 2.3 µl pdN6 (100 ng/µl), and incubated 10 min at 70°C and, then, cooled on ice for 2 min. To start the reaction, 9 µl of reverse transcriptase buffer (5x Superscript II, Invitrogen Life Technologies), 2.3 µl dNTP and 4.5 µl dithiothreitol (0.1 M) were added and incubated first at 25°C for 10 min and then at 42°C for 2 min. The reaction was started by addition of 2.3 µl (460 U) reverse transcriptase Superscript II (Invitrogen Life Technologies) and synthesis was allowed at 42°C for 50 min. The inactivating of reverse transcriptase for 15 min

at 70°C stopped the transcription. 2 µl of this product was directly used for PCR reactions.

3.4.5 Amplification of cDNA (second-strand reaction)

The reaction mixture was made of a final volume of 25µl, including TE buffer 10x, 0.25 U di Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.5 lM of dNTPs, 0.5 lM of forward and reverse primers (see above) and 1µl of cDNA template obtained from the retro-trascription PCR. The primers were designed to amplify a fragment of 810 bp of BDNF gene (shown below) cycling and the conditions were as follows: 94°C for 1 min, followed by 35 cycles of 94 °C for 30 s, 55° C for 30 s and 72 °C for 1 min. A control reaction was done separately with β-actin primers, which yielded a product of 630 bp. The β-actin PCR was performed with a sense primer (5′-CTT TTC ACg gTT ggC CTT Agg gTT-3′) and an antisense primer (5′-AgA TTA CTg CCC Tgg CTC CTA g-3′) at 94 °C for 4 min, followed by 29 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 10 min. As a negative control, DNAse-treated RNA from brain was screened by PCR with BDNF primers to confirm the absence of genomic DNA. This control showed that the PCR product was amplified from cDNA and not from residual genomic DNA in the brain samples.

810 bp cDNA sequence for *N.furzeri* BDNF

ATGACCATCCTGTTCCTTACTATGGTTATTTCATACTTCAGTTGCATGAGAGCTGCGC
CCCTGAGAGACGCCCCGGGCATTCGGGGCCATCGGACGGAAGGCTACTTGGGCGCT
GCTGCGACGACCGCTCGAGGCCACGGGACTCTGCAGAGTGGCGGCGGGCCGGGCCA
GCACGGCGAGCTGCCCTCGCTCACAGACACGTTCGAGCAGGTGATCGAGGAGCTGC
TGGAGGTGGAGGGAGAGGCCGCACAGCTGGGTCAGGGGTCCGACAAGAGCCAGGG
AGGCGGGGGCCCGTTGCCCGTGGTCACCACAGAGAACAAGGATGTCGACCTGTACA
ACTCGCGGGTGATGATCAGCAACCAAGTGCCTTTGGAGCCGCCGTTGCTCTTTCTTC
TGGAGGAATACAAAAACTATCTGGATGCTGCAAACATGTCCATGAGGGTGAGGCGA
CACTCCGACCCCTCACGGCGTGGAGAGCTGAGTGTGTGTGACAGTATTAGCCAGTG
GGTGACAGCTGTGGATAAAAAGACGGCAATAGACATGTCCGGGCAGACAGTTACCG
TCATGGAAAAGGTCCCTGTCCCCAATGGCCAGCTGAAGCAATACTTTTATGAGACCA
AATGCAACCCCATGGGGTACACAAAGGACGGCTGCAGAGGAATAGACAAGCGGCA
TTATAACTCCCAATGCAGGACAACCCAGTCCTACGTGCGAGCGCTCACCATGGATAG

CAAAAAGAAGATTGGCTGGCGGTTTATAAGGATAGACACTTCATGTGTATGCACATT
GACCATTAAAAGAGGGAGAT

3.4.6 Agarose Gel Electrophoresis

RNA and DNA were loaded on 1% agarose gels containing 0.5 μg/ml ethidium bromide in 1 x TAE Buffer and run at 5-10 V/cm. Genomic DNA was loaded on low concentration agarose gels (1%) gel and run slowly (1-2 V/cm) to ensure better separation and to avoid smearing the DNA. The loading dye used was purchased from Bioline. The DNA-HyperLadder I Bioline was used for estimation of molecular weight.

3.4.7 In situ hybridization

3.4.7.1 Synthesis of BDNF riboprobe

a) Cloning of BDNF gene

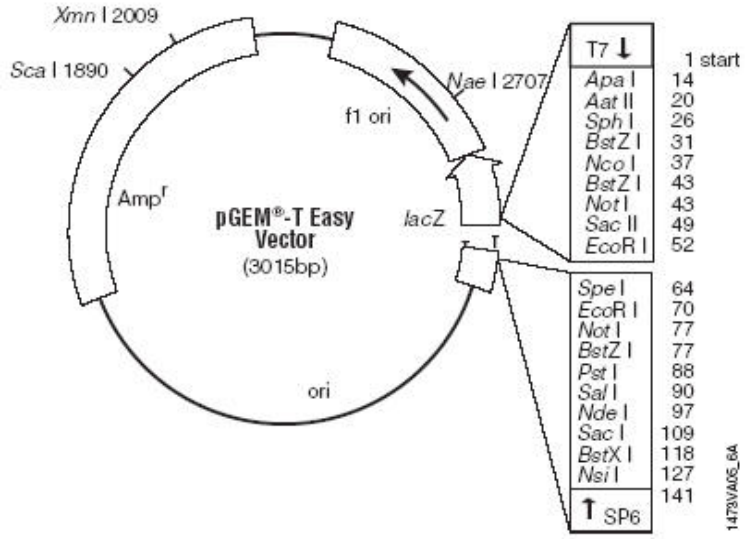
PCR products were cloned using pGEM®-T and Easy Vector Systems Technical Manual (Promega; Madison, WI, USA) following the manufacturer instructions.

b) Cleaning PCR products

PCR products were cleaned up by using the kit NucleoSpin® Extract II (MACHEREY-NAGEL GmbH & Co, Düren, Germany). DNA was purified from contaminations like salts and soluble macromolecular components, which were removed by a simple washing step with ethanolic Wash Buffer NT3. Pure DNA was finally eluted under low ionic strength conditions with slightly alkaline Elution Buffer NE.

c) Ligation of DNA Fragments and PCR Products

Ligation reactions were used to combine vector and insert DNA. For this purpose, purified insert DNA was ligated to dephosphorylated vector DNA using T4 DNA ligase (pGEM-T) according to the supplier's instructions. PCR products (3 μl) were ligated directly into the pGEM-T (Promega) vector according to the manufacturer's instructions.



d) Transformation of ligation reactions into competent cells

The transformation reaction was carried out following the supplier instructions. Ligation reactions were transferred into high-efficiency competent cells (*E. coli* DH5α; Invitrogen), and the appropriate protocol was followed. After heat shock, the bacteria were plated onto LB agar plates containing the appropriate antibiotic to select only the transformed bacteria. Transformants were screened, and the white colonies containing the insert were then cultivated.

e) Colony PCR and Mini-prep

Single bacterial colonies were picked and inoculated in LB medium containing the appropriate antibiotic plates. The bacteria were grown for one to three hours in an orbital rotator at 37°C. 5 µl of bacterial suspension was used as a template in a PCR reaction. All PCR reactions were carried out in a final volume of 20 µl containing 1 x PCR buffer, 1.25 mM MgCl2, 10 pmoles of each primer, 0.1 mM of each dNTP, 1 U of Taq DNA polymerase (QIAGEN). Generally M13 primers or other vector primers like T3 and T7 primers were used.

PCR conditions were as follows : 1 cycle at 95°C per 5 min, at 95°C per 1 min, 30 cycles sub-divided at 54°C per 1.5 min, at 72°C per 1 min, 1 cycle at 72°C 5 min.

Clones that were positive in the PCR were used to inoculate 3 ml of LB medium.

Some of the clones were then used for a mini-preparation, which was carried out by the ZR Plasmid Miniprep™- *Classic* applying the manufacturer instructions.

f) Sequencing

Plasmidic DNA sequencing was carried out at the Laboratory of Genome analysis (Platzer Laboratory), FLI- Leibniz Institute for Age Research. Sequence analysis was carried out using BLAST (Basic Local Alignment Search Tool), accessed through http://genome.fli-leibniz.de/world_nf_blast/world_blast_nf.pl. Alignments of the sequences with several closely related genes were carried out subsequently.

g) In vitro transcription of riboprobes

Antisense and sense RNA probes labeled with digoxigenin (DIG) were generated by in vitro transcription according to the manufacturer's instructions (ROCHE). Plasmids were linearized by digestion. Restriction enzymes used were from New England Biolabs. Digestions were performed using about 200 ng of plasmid in 1 x restriction enzyme digestion buffer and 6 U of restriction enzyme in a total volume of 20 µl. Digestion mixtures were incubated for 1-2 h at the appropriate temperatures for each enzyme as suggested by the manufacturer. Digestion products were controlled by electrophoresis analysis on agarose gel. Successively, protease K (50 µg/ml) and SDS 1% were added to the digested products and an incubation at 45°C per 30 min. After the extraction with phenol-chloroform-isoamylalcohol (PCI = 25:24:1), the aqueous phase was then extracted using 200 µl chloroform. The aqueous phase, containing the DNA, was then ethanol precipitated using 0.3 M sodium acetate and two to three volumes of ice-cold absolute ethanol. Precipitation was allowed at -20°C for 30 min or at -80°C for 10 min. After centrifugation at maximum speed at 4°C for 30 min, the pellets were washed with 70% ethanol air-dried and re-suspended in the appropriate buffer. BDNF RNA riboprobe was generated by linearization with a restriction enzyme .

For transcription, T7 and SP6 polymerase promoter sequences in the vector backbone were utilized. About 100ng of linearized plasmid template was used

for labeling. Transcription buffer and DIG-labeling mixture were added to a final concentration of 1 x. 4 U of RNA.

Polymerase (T7 and SP6) and 20 U of DNAse inhibitor (ROCHE) were also added. The reaction was incubated at 38°C for 2 hours and terminated by addition of 2 µl of EDTA (200 mM, pH 8.0). The RNA transcript was ethanol precipitated and analyzed for size and integrity using agarose gel electrophoresis.

3.4.7.2 LNA probe design

LNA modified DNA oligonucleotides probes, containing an LNA nucleotide at every third position, and labeled at the 59 end only, or at the 59 and 39 ends, with DIG, were supplied by Exiqon Inc. (Vedbaek Denmark). BDNF, NGF, NT-3, NT-4 and NT-6 probes were designed using the Primer3 primer design program (Untergasser *et al.*, 2007) and checked using the LNA Oligo Optimizer tool on the Exiqon website (www.exiqon.com). Each probe sequence was screened against all known *N.furzeri* sequences using BLAST. LNA probes typically show single nucleotide specificity (Kloosterman *et al.*, 2006) (see Table 1). Negative controls were mismatch probes, designed and synthesized by Exiqon Inc. (Fig 3a,b).

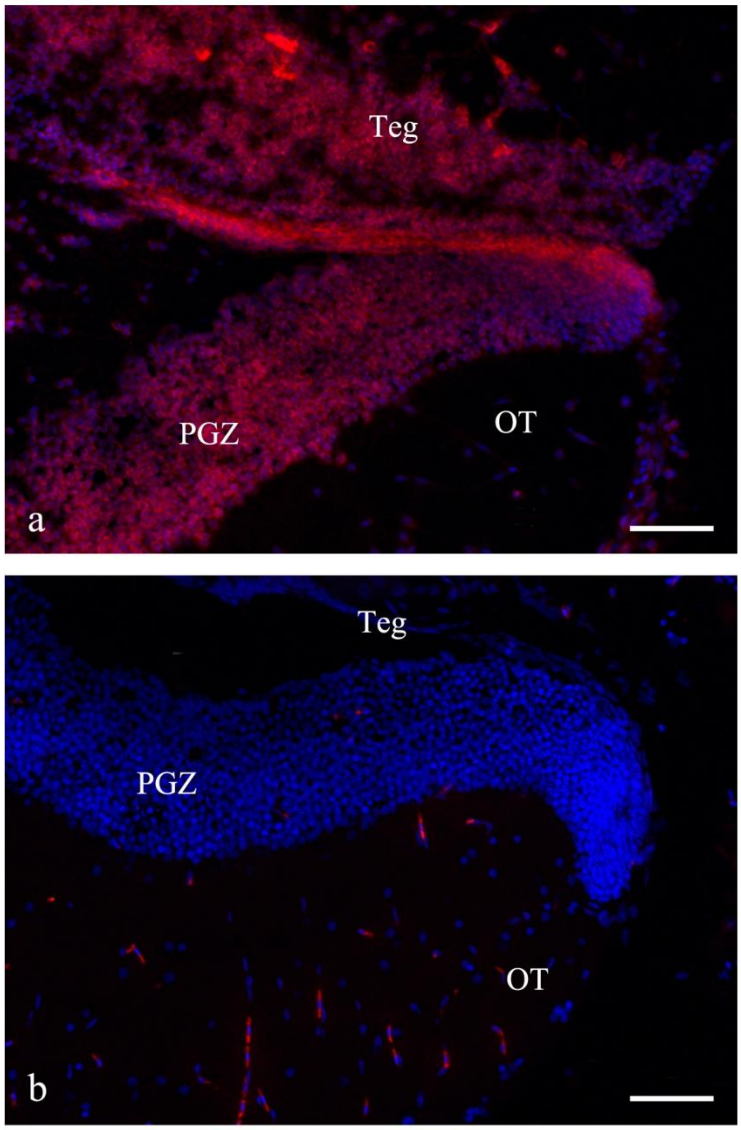


Fig 3. Negative controls: LNA probe vs mismatch probe. a. NT-6 LNA probe labeling in the optic tectum (OT). **b.** NT-6 mismatch LNA probe**.** In blu DAPI counterstaining. Scale bars: a-b = 100 µm.

Table 1. LNA Probes for mRNA detection

Target mRNA	5'-Mod	Synthesis sequence (5'-3')	3'-Mod
NGF	DIG	ACGCTAACACTTTCACACACAGA	DIG
BDNF	DIG	ATACTGTCACACACACTCAGCT	DIG
NT-3	DIG	TGAGAGAGTTTAAAGGGTCAGG	DIG
NT-4	DIG	GCAAAACAACAAGACCTCAACAC	DIG
NT-6	DIG	TTGTCTCCTGCTGTCCTGCTCTG	DIG
*mut NT-6	DIG	TTGTCTC**TC**GCTG**CT**CTGCT**TC**G	DIG

* mutations are shown bolded

Target NGF mRNA

ATGAGGTCATCCATGCTGGTCCTGTTCCTCTTCTTCAGTGCCCAGGTAGTGGCCAAC
ATCAGAGGGGACTCGAGCAGCCCCACAACACTGCAGCAGATCAATGCCCACTCCAT
CCCCTCTGTGGACCCAAGCCTCTTCATCAAGCGCCACCATCTCTCCCCCAGGGTGCT
TTTCAGCTCTCAACCTCCTGATGATGAACCAGCAGGGTCGCAAGGGGGCGGCAGAA
GGACCCGCAGGCGAGCAGGACAACCTCAGCACCGCGGGGTATACTCTGTGTGTGAA
AGTGTTAGCGTCTGGGTGGGCAACAAGACCAAAGCCACAGACATCTCGGGCAATGA
GGTGACGGTGCTCCCCGATGTGAACATCAACAATGTCAACAAGAAGCAGTATTTCTT
CGAGACAACGTGTCACAGTTCTCGACCTGGCAGCTCGGGCTGTTTAGGAATCGATGC
GAGACACTGGAACTCCTACTGCACCAACTCACACACTTTTGTTCGAGCGTTGACTTC
ATTCAATGACATGGTGGCGTGGAGGCTCATACGCATCAATGTGGCATGTGTGTGTGT
GCTCAGCCGCAAGTCGTGGCGACAGTGA

Target BDNF mRNA

ATGACCATCCTGTTCCTTACTATGGTTATTTCATACTTCAGTTGCATGAGAGCTGCGC
CCCTGAGAGACGCCCCGGGCATTCGGGGCCATCGGACGGAAGGCTACTTGGGCGCT
GCTGCGACGACCGCTCGAGGCCACGGGACTCTGCAGAGTGGCGGCGGGCCGGGCCA
GCACGGCGAGCTGCCCTCGCTCACAGACACGTTCGAGCAGGTGATCGAGGAGCTGC
TGGAGGTGGAGGGAGAGGCCGCACAGCTGGGTCAGGGGTCCGACAAGAGCCAGGG
AGGCGGGGGCCCGTTGCCCGTGGTCACCACAGAGAACAAGGATGTCGACCTGTACA
ACTCGCGGGTGATGATCAGCAACCAAGTGCCTTTGGAGCCGCCGTTGCTCTTTCTTC
TGGAGGAATACAAAAACTATCTGGATGCTGCAAACATGTCCATGAGGGTGAGGCGA
CACTCCGACCCCTCACGGCGTGGAGAGCTGAGTGTGTGTGACAGTATTAGCCAGTG

GGTGACAGCTGTGGATAAAAAGACGGCAATAGACATGTCCGGGCAGACAGTTACCG
TCATGGAAAAGGTCCCTGTCCCCAATGGCCAGCTGAAGCAATACTTTTATGAGACCA
AATGCAACCCCATGGGGTACACAAAGGACGGCTGCAGAGGAATAGACAAGCGGCA
TTATAACTCCCAATGCAGGACAACCCAGTCCTACGTGCGAGCGCTCACCATGGATAG
CAAAAAGAAGATTGGCTGGCGGTTTATAAGGATAGACACTTCATGTGTATGCACATT
GACCATTAAAAGAGGGAGATAG

Target NT-3 mRNA

ATGTCCATCCTGCTGTATATGATGTTCCTTGTGTACCTCTATGGTATCCAGGCAACCA
ACATGAACAGCAGCCACCATGGACAGCAGCAGCAGCCAAGTCCTGACCCTTTAAAC
TCTCTCATAATCCAGCTGCTGCAGGCTGATCTGACCAGAGGGAGGACCCGGGGGAA
CTACAGCCAGCAGGGAAAAAGCAGGGATATGGCCCTGCAGGAAACACTGCCTCCTC
TCCCTGATGTGGAGCACTGGGGATCCTGGGGTCGTAGCGGCGGTAGCAGTGACGGT
GCCGTCGAGCAACAGGTGATGCTGTTAAACTCAGATCTCCTCAGGAAGCAGAAGCA
ATACAGTTCACCTCGGGTGCTGCTGAGCGACCGGCCACCACTACAGCCACCGCCGCT
CTACTCAGCTGATGACTTTGTAAGCGGTGGACCAGACAGGGGCGCAATGGGGAACA
GGACGCGTAGGAAGCGCAACACTGGACACAGGAGCTACCGTGGGGAATATTCTGTC
TGCGACAGTGAGAGCCAGTGGGTGACGGACAAAGACCACGCCGTGGACACCAAAG
GAGAACCCGTGATTGTTCTAGCCAAAATCAAAACTAGCGCCACGCAGGACGTCAGA
CAATTCTTTTACGAGACGCGCTGTCGGACCCCGGGAACCAGTAAAGGAGGCTGCAG
GGGGATCGATGACAAGAACTGGAATTCTCAATGCAAGACGACACAGACGTATGTCC
GAGCCCTAACACAGGCTCAGAATAAAGTGGGATGGAGGTGGATACGCATAGACACT
TCCTGTGTGTGTGCATTGTCACGGAAACGCCGTAAGACGTAA

Target NT-4 mRNA

GGATACAAAGATCTTTTATTACATGTCCGACTGAACACAACAGCAGGTTTTCTTCAG
GTGTCACTTAATTTACAAACCACATAGTTTCATGTTCTTGAACCACATTTGAGGAGT
CCCACATACGCAATCTGACACAAATTATAGTTTGTTCTGAATACATTCCCCTTTGCA
ACATTCATAAGAGTTTGGAGCTGTAAAATAAACTGTCAGTAGGTGGCGCTCTTTGTT
TTTAGAAAAACACAGCAAGCCAAAGGCTGGAATTATCATCAGACACTGGAGAGACT
GCTGCGTGAAGACGTCTTCTATAAATACACACTGAAATACATAAGGGTATACTGGTG
TCATGTTATACACACGTTAAGGAATATATTAATATCAAAGACATGCTTACATAATAA
TCAAAGATAACTTAGATTATGAAAAGTAGGTCATACAAAACAGTACTTCTTGATGTA
AAATCTATAAAACAAATTTCCCTAAGGTCTAGTGTTGAGGTCTTGTTGTTTTGCAAA
AGTCTCATGGTTGAGGGAGGGGAAATCCAGAGATGTGGGGGAAAGTGAACAAGAG
CAGGGCTACTGTACACAGAAGAACATCTCAGTTTCTCTGTGCCCCCATTTTATCCTCT
CCCCCGGGTCAAAACCTCCTTCTTTGTTCTGGACAGCAGCACGCAGACGCAGGACGA
GTCGATTCGGATCCACTGCCATCCGATTTGGTTCCTTTCGTTTCTAATTAGTGCTCGA

ACAAACGACTGCTTGACCTTGCACTCGCTCCTCCACTGTTTTTTATCCACACCCAAAC
ATCCAGCTCCGGATACGCCCGTCCCCACCGACCTCCCTGT

Target NT-6 mRNA

CTGGTTTCTGCTCCTCCTGATCGGCGTCCAGGCTGTACTGAACATGGGTGGTGGGTT
CGGCCCGAAGTGCTGGGGCGGCCAATCGCAGAGCAGGACAGCAGGAGACAACAGC
CAGGGACCACCTTTCTCAGGACCACACTTTGCAGCAGCATCATAGGACCACCCACCA
CAGGACCAAGAGGCCTCATCGAGCAGCTTCAAACACACAGAACAGGAGTCCAGTTG
TTGGGCGTTCCGCTGCGGGTTCCTCATTCGACCTTTCAAGTCCAGTGGTAGACCCCA
AGCTCTTCTCTAAGAGACGATACCGTCCCTCGCCCCGTGTTGTCTTCAGTGAGGTGC
CCCCATCCCACGATGCCCTGGAAGGAGAGGGCTATGACATTGAAGGAGTCAAGGAG
GTGAGGGTGCGGCGCAGAGCAGGATCACACATCATGCACAGAGGGGAGTACTCAGT
GTGTGACAGCATAAACACCTGGGTGGGAAACCTGACACGAGCCACAGACATAGCTG
GGAATGAAGTGACCGTGCTCCCCAACGTTACAATCAACAACGTGGTCAAGAAACAG
TTCTTCTATGAGACCACCTGTCGATCCCCCACACACAGGGGCACTGGGGCTGCGAAT
GGGAGCCGGCCTGGAGGACGGGGCGGAAAACAAGGCTCAAAATCCAGCACCTCAG
GTTGCCTTGGCATTGACAGTCGCCACTGGAACTCCCACTGCACCAACACA

3.5 Histological Techniques

3.5.1 ISH on brain cryosections

In situ hybridization was used to determine the cellular localization of specific messages within cell populations of the central nervous system of *N. furzeri*. Two appropriate protocols were followed, respectively for riboprobe and LNA probes.

a) ISH for BDNF riboprobe

The brains were rapidly excised from anesthetized animals, fixed in PFA 4% for 24 hours at 4°C. Successively, brains were incubated in saccharose solution at 30% overnight at 4°C and saccharose solution at 20% overnight at 4°C. The brains, then, were embedded in cryomounting and frozen at –80°C. Serially transversal, horizontal and sagittal sections were cut at cryostat (Leica).

Brain sections of 14 μm thickness were washed in PBS 0.1% Tween 20 (PBT). All buffers are listed in Table 2.

The permeabilization consisted of incubation for 5 min in 2.3% sodium borohydride in 0.1 M Tris–HCl (pH 7.5), followed by 1 mg/ml Proteinase K (Sigma–Aldrich) for 10 min at room temperature (RT). After washing in glycine and PBT, the sections were fixed in 4% PFA in PBS for 15 min at RT. Thereafter, the prehybridization was carried out in a hybridization solution containing 50% formamide, 0.5% SSC, 500 μg/ml Heparin, 50 μg/ml yeast RNA, 0.1% Tween 20 at 55 °C for 1 h. Probes were denatured for 10 min at 80 °C and sections were then incubated, in hybridization solution containing either antisense and sense BDNF probe at a concentration of 2 ng/μg, overnight at 55°C. RNAse free conditions were kept during all prehybridization and hybridization steps. Post-hybridization washes were carried out at 55°C as follows: 2 x 20 min in 1x SSC, 2 x 10 min in 0.5 x SSC, and then in PBT at RT. The sections were blocked in blocking solution containing 10% normal goat serum and blocking reagent (Roche, Germany) for 1 h at RT. After, sections were incubated in fresh blocking buffer containing a 1:2000 dilution of anti-digoxigenin Fab fragments conjugated with alkaline posphatase (Roche) and blocking solution, overnight at 4°C. The sections were washed in PBT 5 x 5 min

and in PBT + Levamisol (5mM) 3 x 5 at RT. The chromogenic reactions was carried out by using Fast Red tablets (Sigma-Aldrich) in Tris buffer and incubating the slides at RT for 3-4 h, in the dark. After the signal was developed, the sections were washed in PBT 3 x 5 min at RT, and mounted with Vectashield (VECTOR) contains DAPI that is used as counterstaining for the nuclei.

b) ISH for LNA probes

The protocol followed for ISH using LNA probes included only few different steps compared to that applied for the BDNF riboprobe. The prehybridization and the hybridization steps were carried out at 42°C. Post-hybridization washes were carried out as follows: 2 x 20 min in 1x SSC at room temperature, 2 x 10 min in 0.5 x SSC at RT.

Table 2. ***Buffers preparation***

TAE 50x	Tris Base 242 gr Acetic Acid 57.1 mL EDTA 0.5 M 100mL
TBE 10x (1 l)	Tris 108gr Boric acid 55gr EDTA 0,5 M 40ml (pH 8,0)
TE	Tris-HCl (pH 8,0) 10mM EDTA 1mM
TRIS 10x pH 7.6	Trizma base 61gr Distilled H_2O 1 l
LB –Luria Bertani medium (1 l)	NaCl 10gr Bacto-Triptone 10gr Yeast extract 5gr
SOB	Trypton 20gr Yeast extract 5gr NaCl 5 M KCl 1 M H_2O up to 1 liter
SOC	Add to SOB for 1 liter $MgSO_4$ 1 M 10ml $MgCl_2$ 10ml Glucosio 1M 20ml

DEPC H_20 0,1% (1 l)	Distilled H_20 1 l Diethylpirocarbonate 100 µl
PBS Tablets Sigma-Aldrich	DEPC H_20 1l PBS tablets 5
PBST	DEPC H_20 200 µl PBS tablet 1 Tween-20 100 µl
Glycine in PBT	DEPC H_20 200 µl Glycine 2 gr
LB –Luria Bertani medium (1 l)	NaCl 10 gr Bacto-Triptone 10 gr Yeast extract 5 gr
SSC 20% pH 7.0	DEPC H_20 500 ml NaCl 3 M Na_3Citrate 300 mM
Blocking solution	PBS 1x Goat serum 10% (heat inactivated) Blocking Reagent 0,5% Tween-20 0,1% H_20 up to the final volume
Hybridization Buffer	Formamide 50% SSC 5x Tween-20 0,1% Citric-Acid 9,2mM Heparin 50µg/ml Yeast t-RNA 500µg/ml DEPC H_20 up to the final volume

3.5.2 IHC

a) Antibody Staining on Fresh Frozen Cryostat Sections

The collectioning samples and subsequent treatment procedures have been described in the paragraph n° 3.5.1.

Sections of 14 µm thickness were cut in transverse, horizontal and sagittal orientation. Sections were fixed in 4% PFA for 10-20 min at RT and washed three times for 10 min at RT each in PBS 1x (pH 7.5). Tissue in the slides was then dried by incubation in aceton for 15 min at RT. The slides were washed 3 x for 5 min in PBST (PBS + 0.1% TritonX-100). Then, they were placed in target retrieval solution (Citric buffer pH 7.4) brought to boil using the microwave, and then gently boiled for 10 min at 10% power, left in the solution to cool for 30 min. Sections were washed with PBS and incubated for 1 h at RT in a solution of PBS with 20% NGS (S1000; VECTOR Lab; Burlingame, CA), 1% BSA, and 0.1% TritonX-100. Primary antibodies are shown in Table 3. Then, they were incubated in a humid chamber for 24 h at 4°C with each of primary antibody diluted with PBS containing 0.2% TritonX-100, 0.1% bovine serum albumin, and 4% normal goat serum. Incubation with primary antibody was carried out overnight at 4°C. After incubation, the sections were washed in PBS 1x and incubated with EnVision for 30 min at RT. After washing, the immunoreactive sites were visualized using a fresh solution of 10 µg of DAB in 15 ml of a 0.5 M Tris buffer, pH 7.6, containing 1.5 ml of 0.03% H_2O_2.

b) Antibody staining on paraffin sections

After anesthesia, animals were decapitated and the heads were fixed in Bouin's fluid for 24 h at RT, washed in alcohol 50% and then progressively dehydrated and embedded in paraffin wax.

Microtomical sections of 7 µm thickness were cut in transverse, horizontal and sagittal orientation. After dewaxing in xylene and dehydrating in progressively diluted alcohols, serial sections were treated with 3% H_2O_2 per 20 min, washed with PBS 1x and incubated in a humid chamber for 24 h at 4°C with each of primary antibody diluted with PBS 1x containing 0.2% TritonX-100, 0.1% bovine serum albumin, and 4% normal goat serum (NGS).

Primary antibodies are shown in Table 3. After incubation, the sections were washed in PBS and treated by using EnVision method: the sections were incubated with EnVision for 30 min at RT. After washing in PBS 1x, the immunoreactive sites were visualized using a fresh solution of 10 µg of 3,30-diaminobenzidine tetrahydrochloride (DAB) (D5905; Sigma-Aldrich) in 15 ml of a 0.5 M Tris buffer, pH 7.6, containing 1.5 ml of 0.03% H_2O_2.

c) Controls

The specificity of the each immunohistochemical reaction was checked in repeated trials as follows: 1) substitution of either the primary antibody or the antirabbit IgG or the EnVision by PBS or non-immune serum; 2) preabsorption of the primary antibodies with homologous antigens (up to 50 mg/ml antiserum in the final dilution) or correlated antigens (up to 100 mg/ml antiserum in the final dilution). The peptides used in the preabsorption were sc-548P, sc-546P, sc-547P, sc-545P (Santa Cruz) for NGF, BDNF, NT-3, NT-4 respectively.

Table 3. ***Primary antisera***

Antisera	*Source*	*Host*	*Characteristics*	*Dilution*
BDNF	sc-546 Santa Cruz Biotechnology	Rabbit	Internal region of BDNF of human origin	1:150
BDNF	ab6201 Abcam	Rabbit	Recombinant full length protein (Human)	1:150
NGF	sc-548 Santa Cruz Biotechnology	Rabbit	N-terminus of the mature chain of NGF of human origin	1:150
NT-3	sc-547 Santa Cruz Biotechnology	Rabbit	internal region of NT-3 of human origin	1:150
NT-4	sc-545 Santa Cruz Biotechnology	Rabbit	internal region of NT-4 of human origin	1:150
Anti-Digoxigenin-AP FAB Fragments	Roche	Sheep		1:2000

3.5.3 Histochemical Techniques

Luxol Fast-Bleu/Cresyl Violet Staining

The collectioning samples and subsequent treatment procedures have been described in the paragraph n° 3.5.2b. Microtomical sections of 7 µm thickness were cut in transverse, horizontal and sagittal orientation and employed for the Luxol Fast-Bleu/Cresyl Violet staining.

After removing paraffin, the sections were rinsed with distilled water, and immersed in acetic acid solution (approximately 20 drops of 10% acetic acid in 100 ml of distillated water) for 5 min, and in 95% ethanol. The sections were, then, incubated in Luxol Fast Blue MSB (LFB; Chroma-Gesellschaft, Könger, Germany) solution (1g LFB in 1000 ml of 95 % ethanol) for 24 h at 37°C. After rinsing in 95% ethanol, followed by distilled water, they were differentiated in 0,05% lithium carbonate for few seconds at RT, and immersed in 70% ethanol 5 times. The sections were subsequently incubated in Cresyl Violet (CV; Katayamakagaku, Tokyo, Japan) solution (0,1 g CV and a few drops of 10% acetic acid in 10 ml of distilled water, then filtered prior to incubation) for approximately 30 min at RT. After rinsing in distilled water, they were dehydrated with ethanol series, cleared with xylene, and coverslipped.

3.6 Phylogenetic Analysis

Phylogenetic analysis was performed by MEGA5 (Tamura *et al.*, 2011). All genes and amino acid sequences were downloaded from ENSEMBL aligned using ClustaW, and the alignment manually inspected. Several different algorithms and substitutions models were tested and all retrieved the same topology. Results of the Neighbough-Joining Method (NJ) are illustrated. Interior branch test was used to calculate the support for the branches.

4. RESULTS

4.1 *Atlas of the brain of Nothobranchius furzeri*

Schematic drawings (Fig 4) of the macroscopical aspect of the encephalon of *N. furzeri* have been done, since the brain structures of teleosts presents enormous interspecific diversity with regards to the morphology (Ito, 1978; Nieuwenhuys *et al.*, 1997). The diversity exists even among strains of the same species (Ishikawa *et al.*, 1999). For example, inbred strains of medaka with different genotypes possess large variation in gross brain morphology (Ishikawa *et al.*, 1999). Furthermore, taking into consideration the high interspecificity among fish and some peculiar features, which were noticed during the experimental procedures, an atlas of the CNS of *N. furzeri*, has been schematically constructed (Fig 4,5a-y), indicating the main encephalic regions in serial transverse sections. The abbreviations list of figured is annexed. This abbreviations list will be apllied also to ISH and IHC results.

The used strain for the description is an inbred strain, long-lived phenotype.

The brain structures were identified mainly based on comparative and hodological information on other Cyprinodontiformes, i.e. *Fundulus heteroclitus* (Peter *et al.*, 1975); *Xiphophorus helleri* (Anken and Rahmann; 1994), and Atherinomorphes, i.e. *Oryzia latipes* (Ishikawa *et al.*, 1999).

The nomenclature of the identified nuclei and the fiber tracts corresponds to Anken and Rahmann (1994) and Nieuwenhuys (1997).

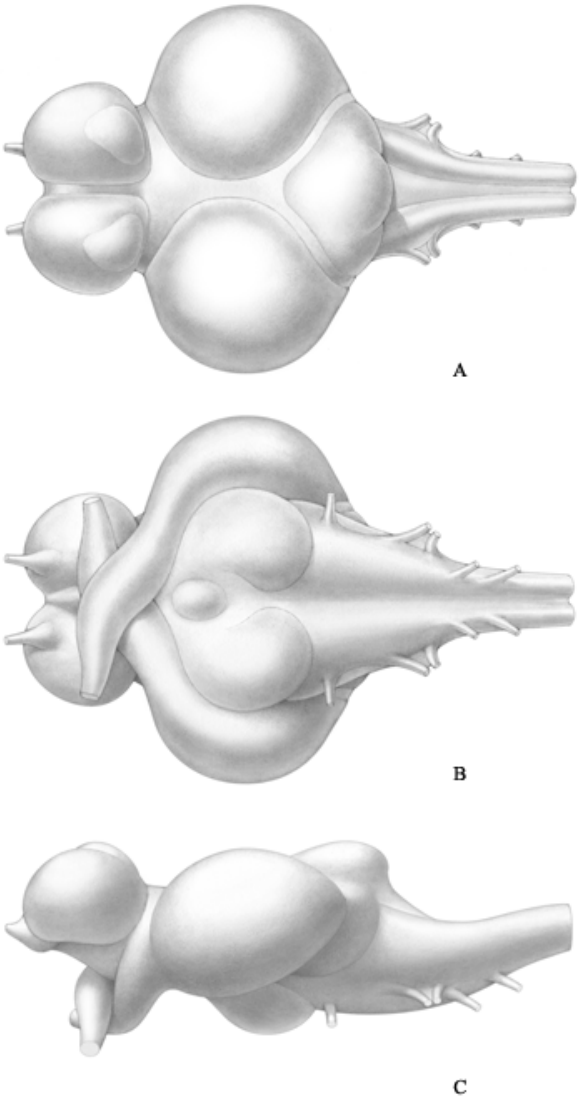


Fig 4. Dorsal (A), ventral (B) and left lateral (C) view of the gross anatomy of brain of *N. furzeri.* Drawings by Massimo Demma ©.

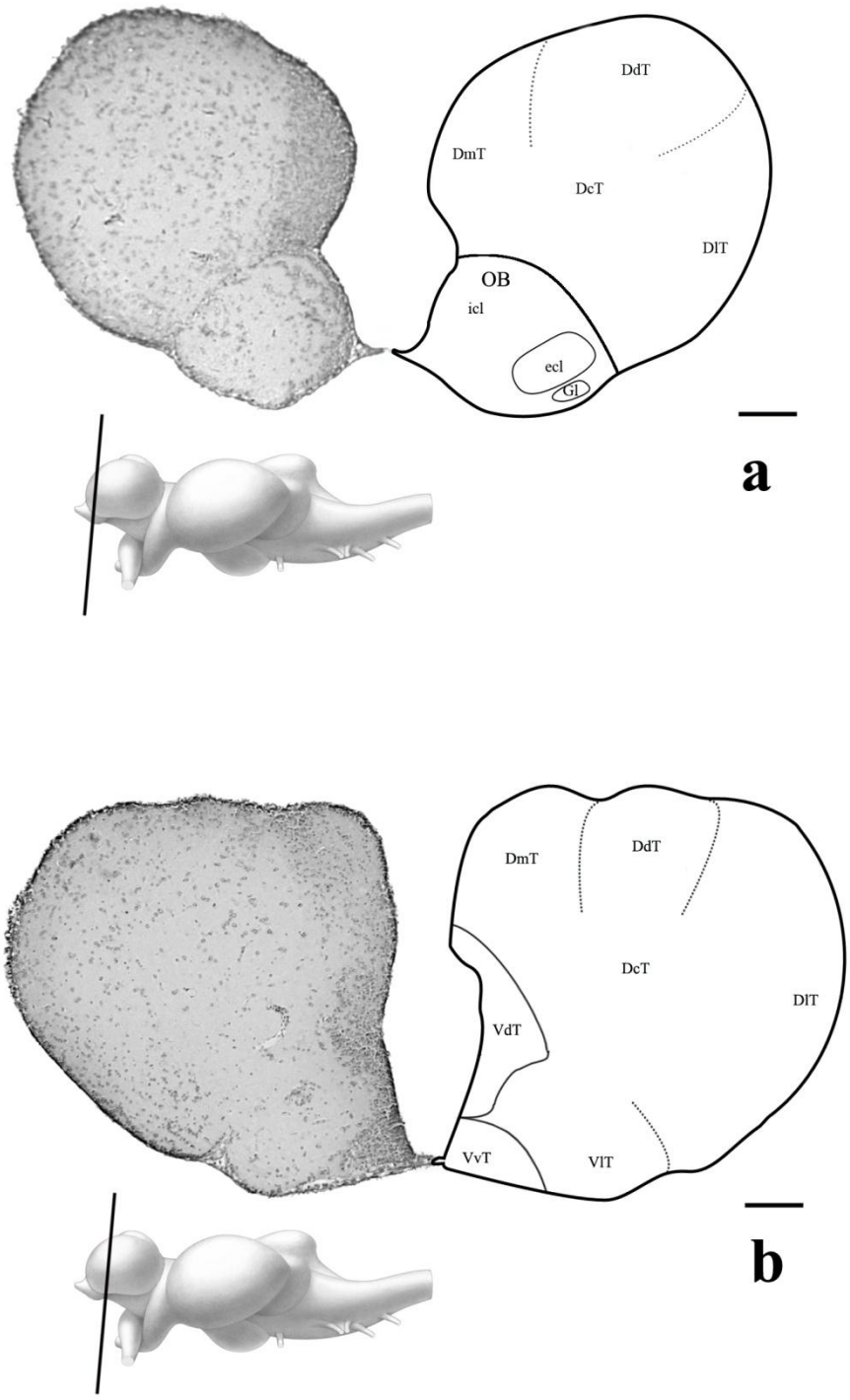


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

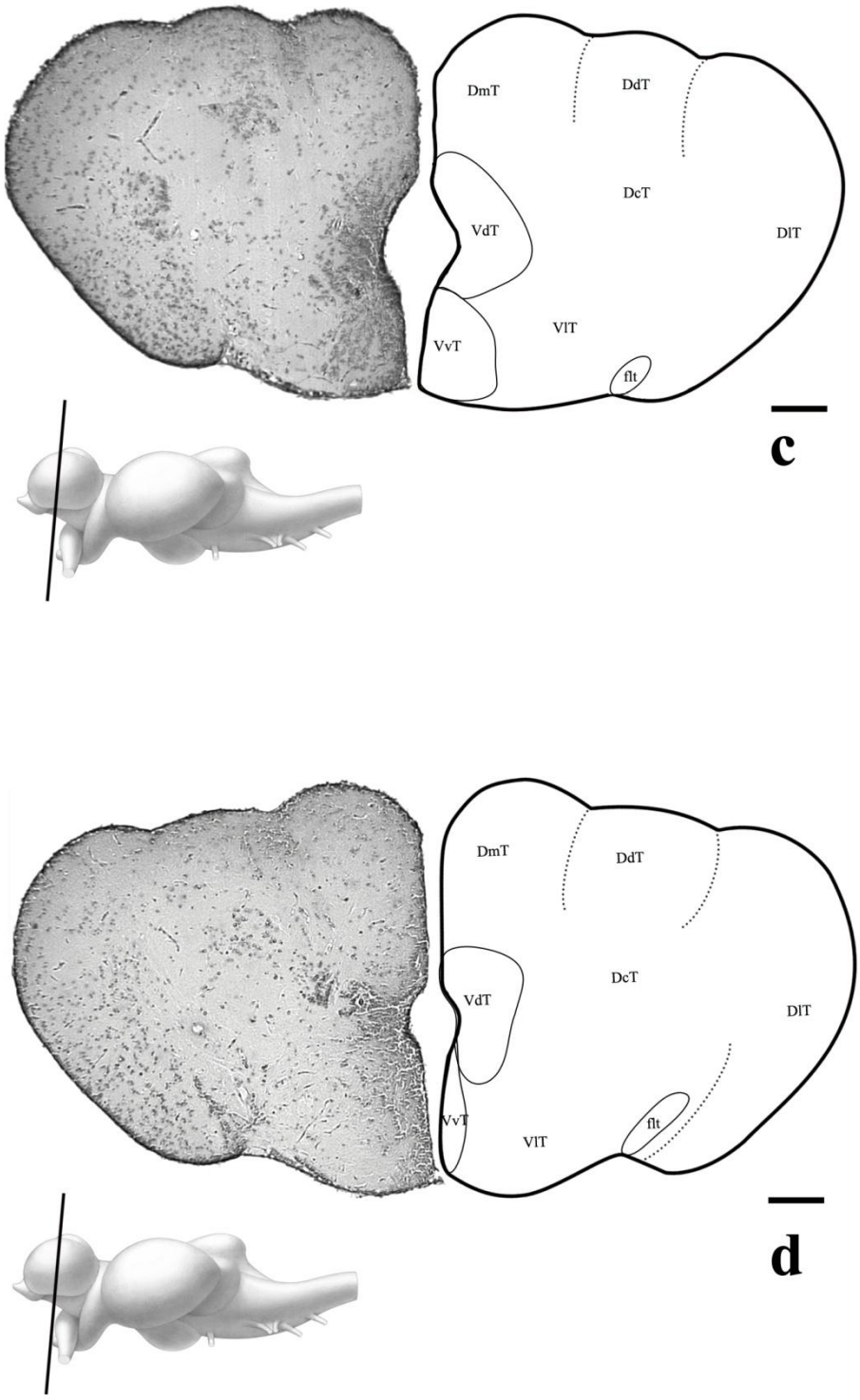


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

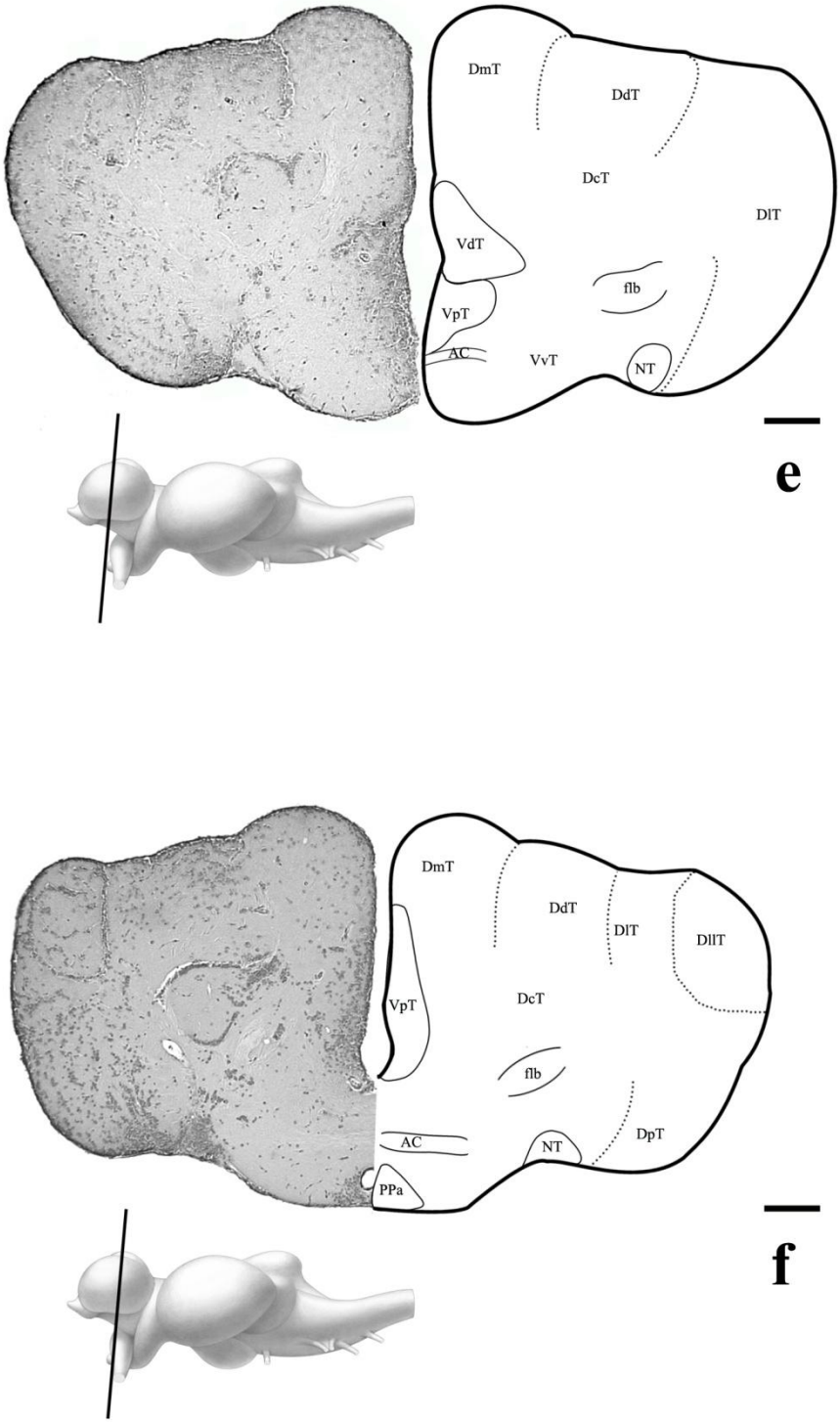


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

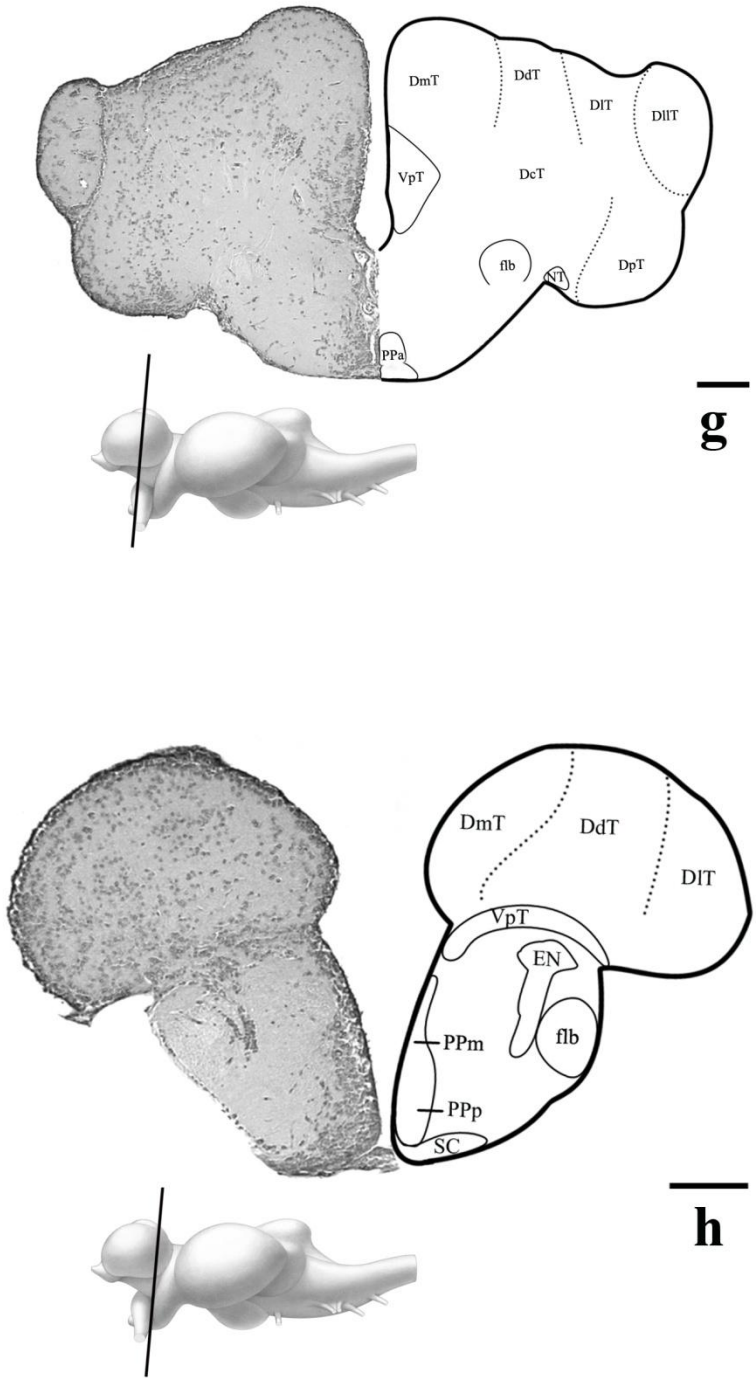


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

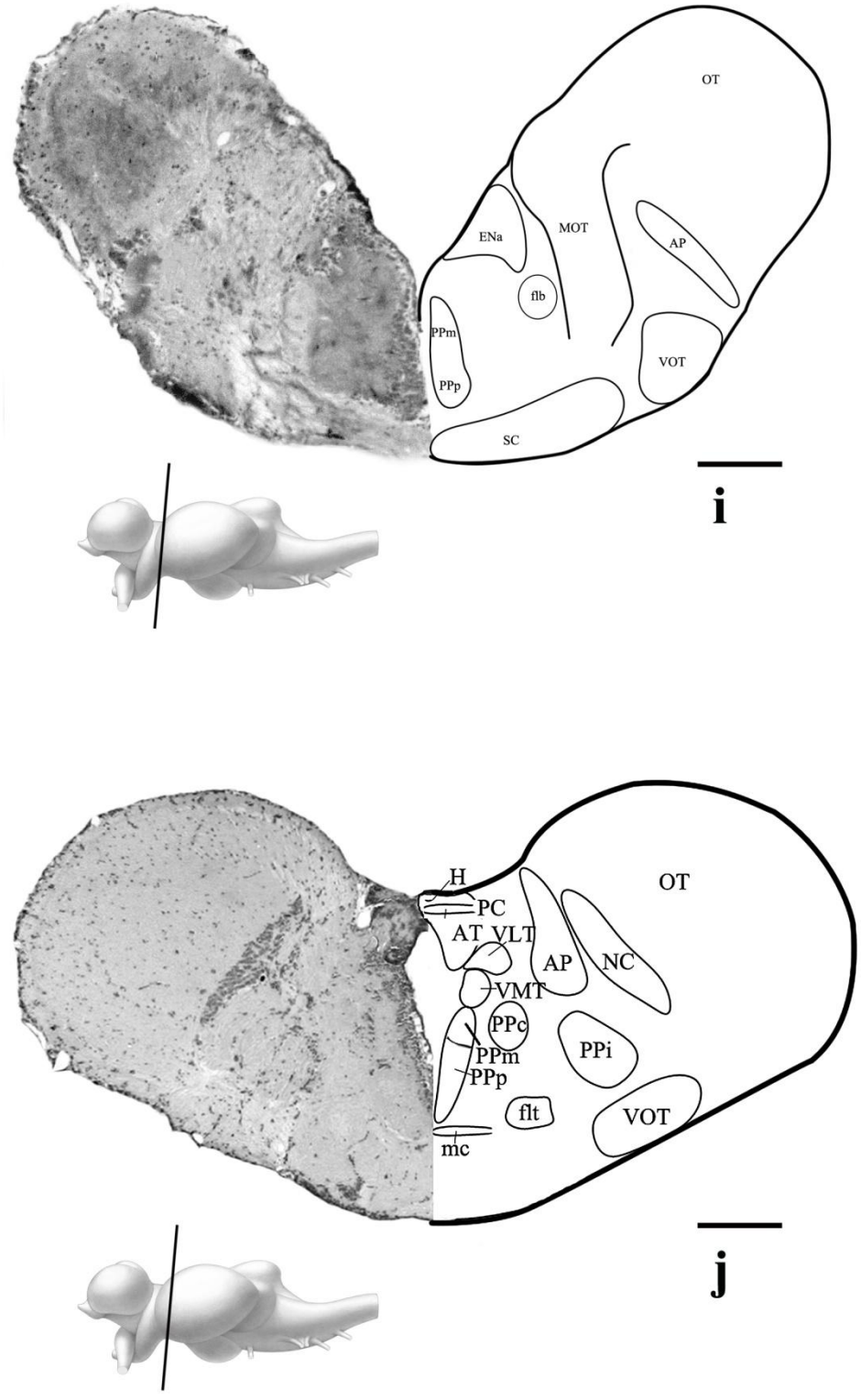


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

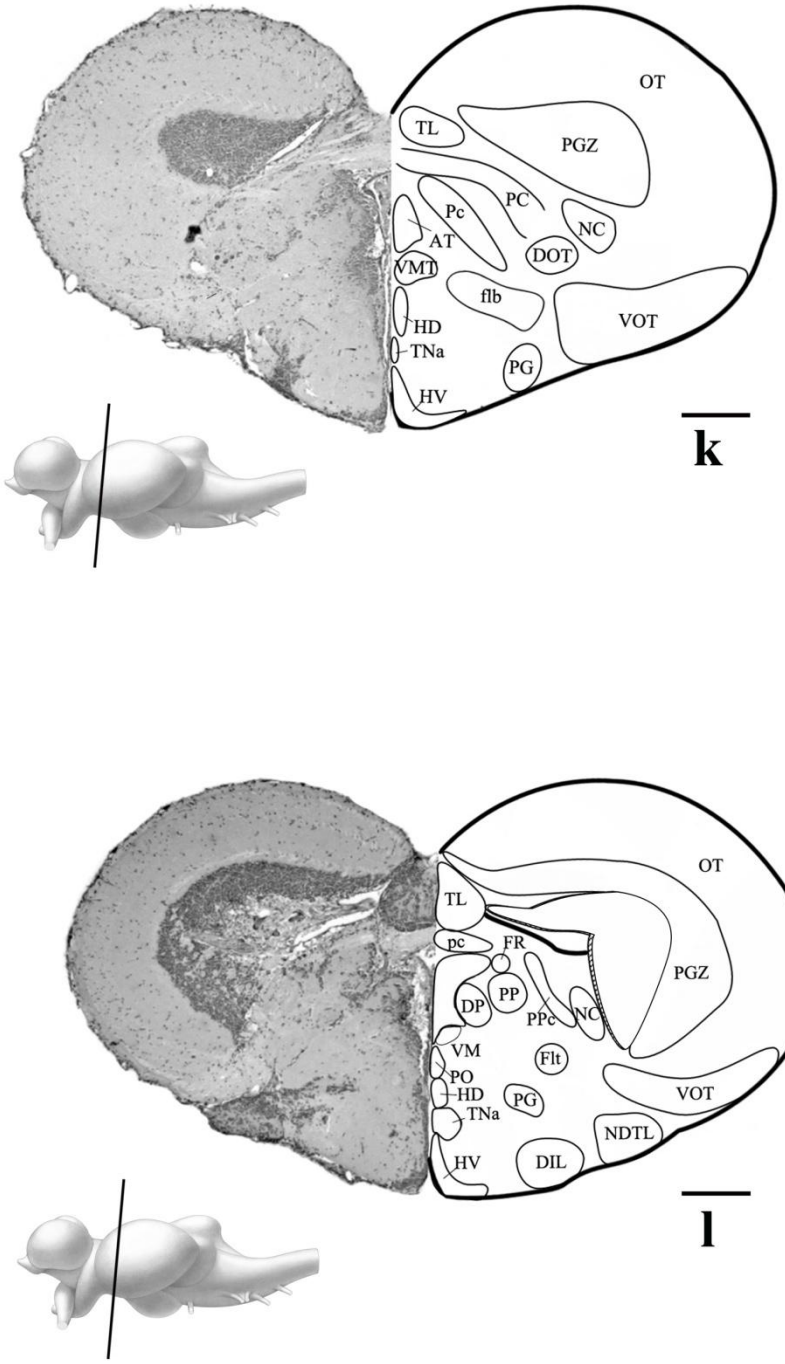


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

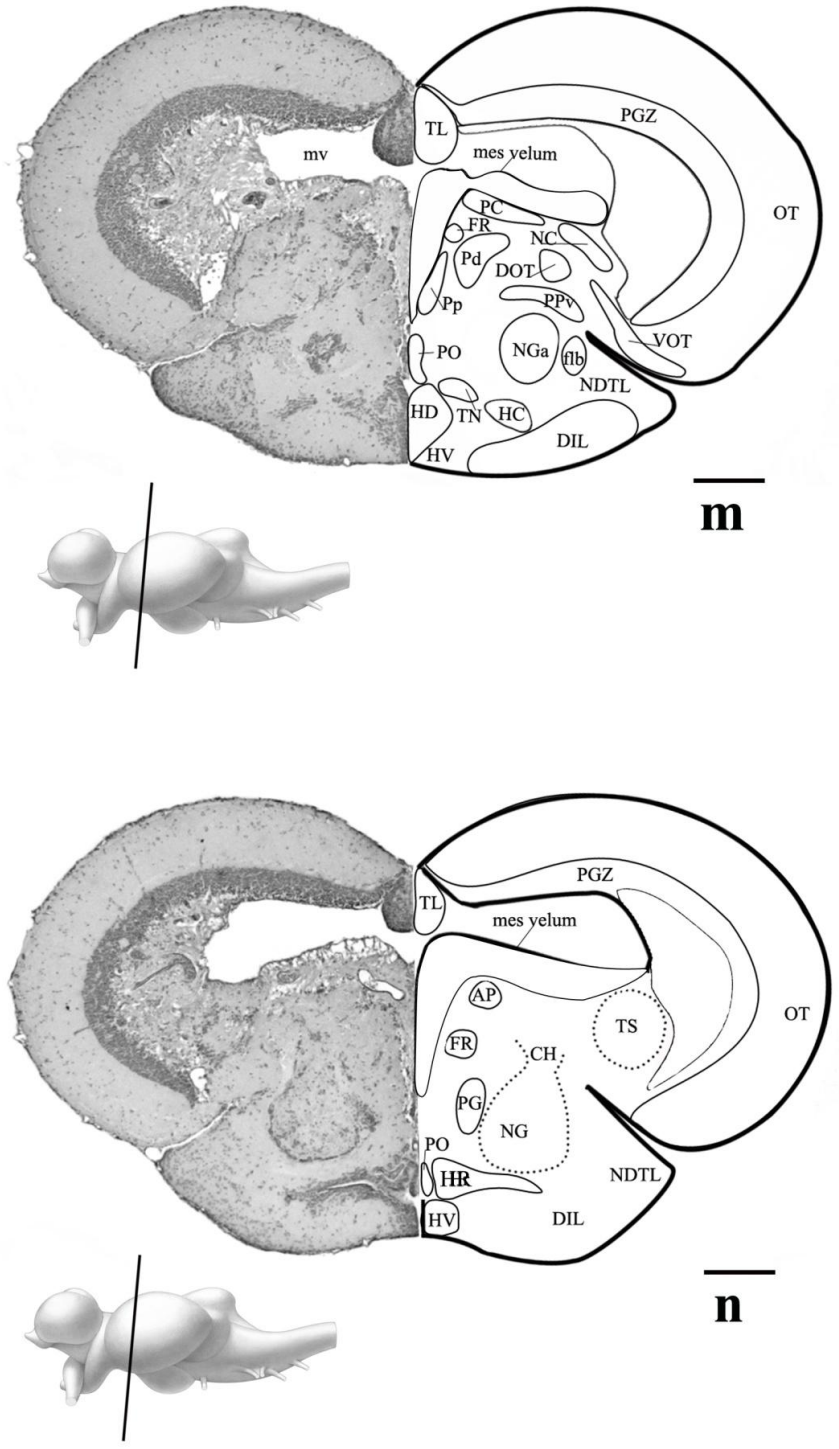


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

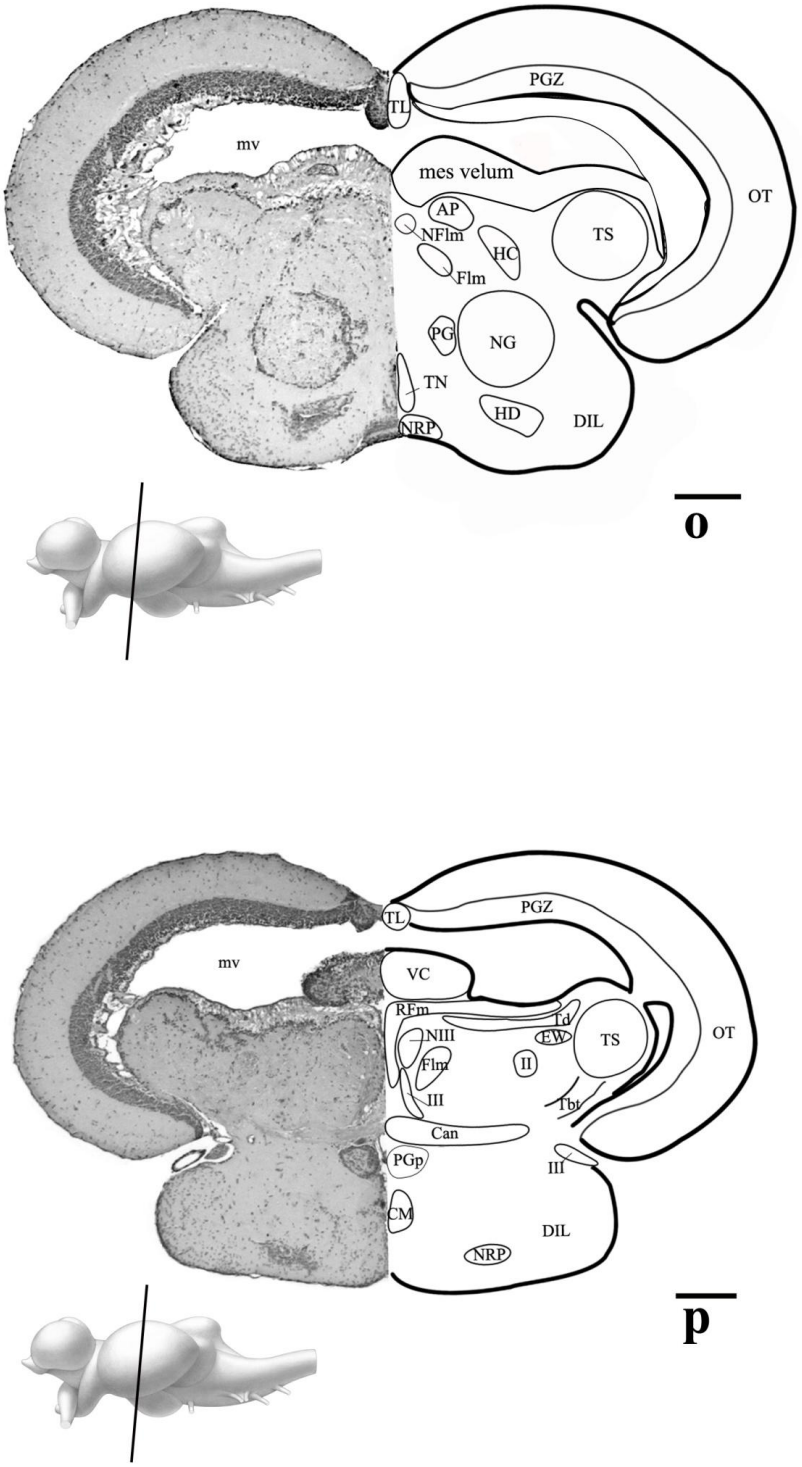


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

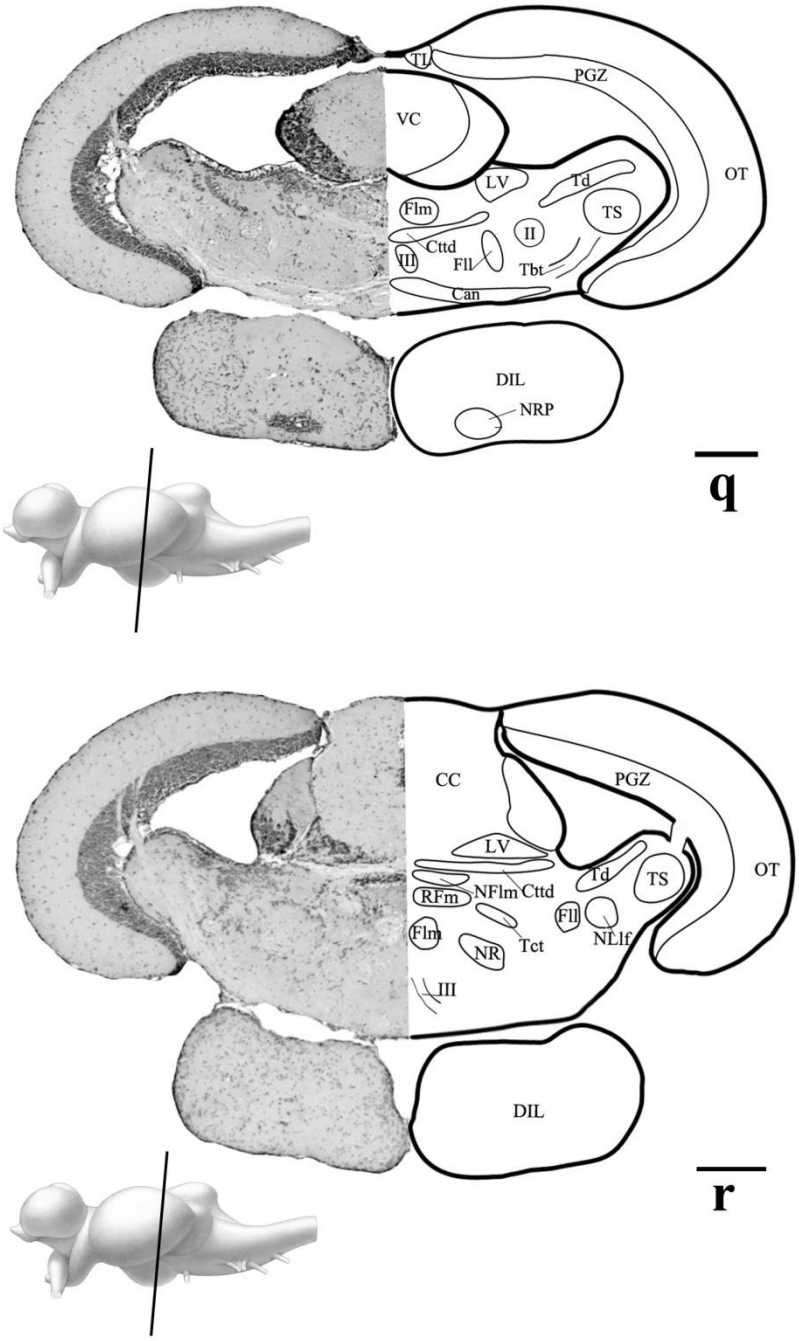


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

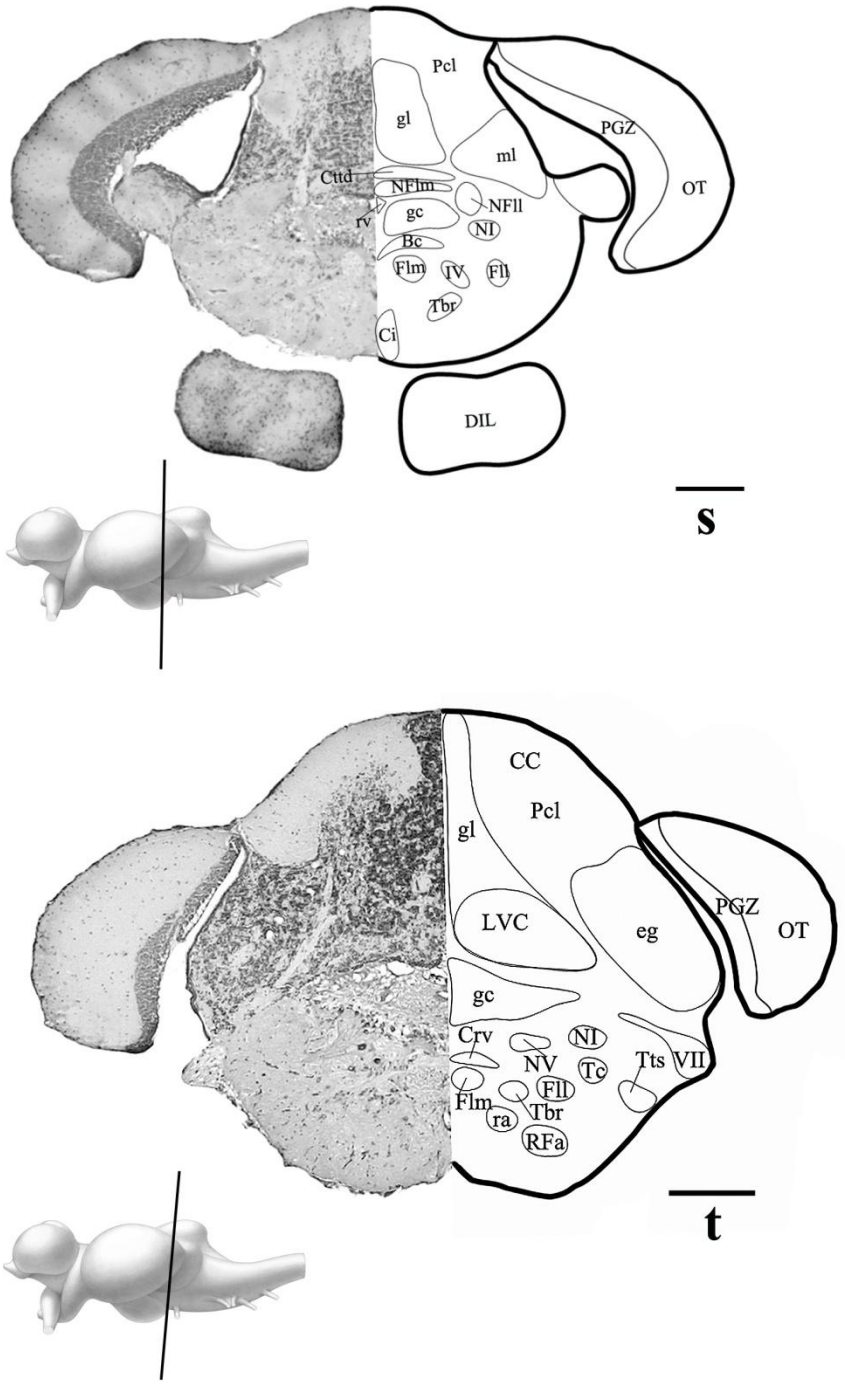


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

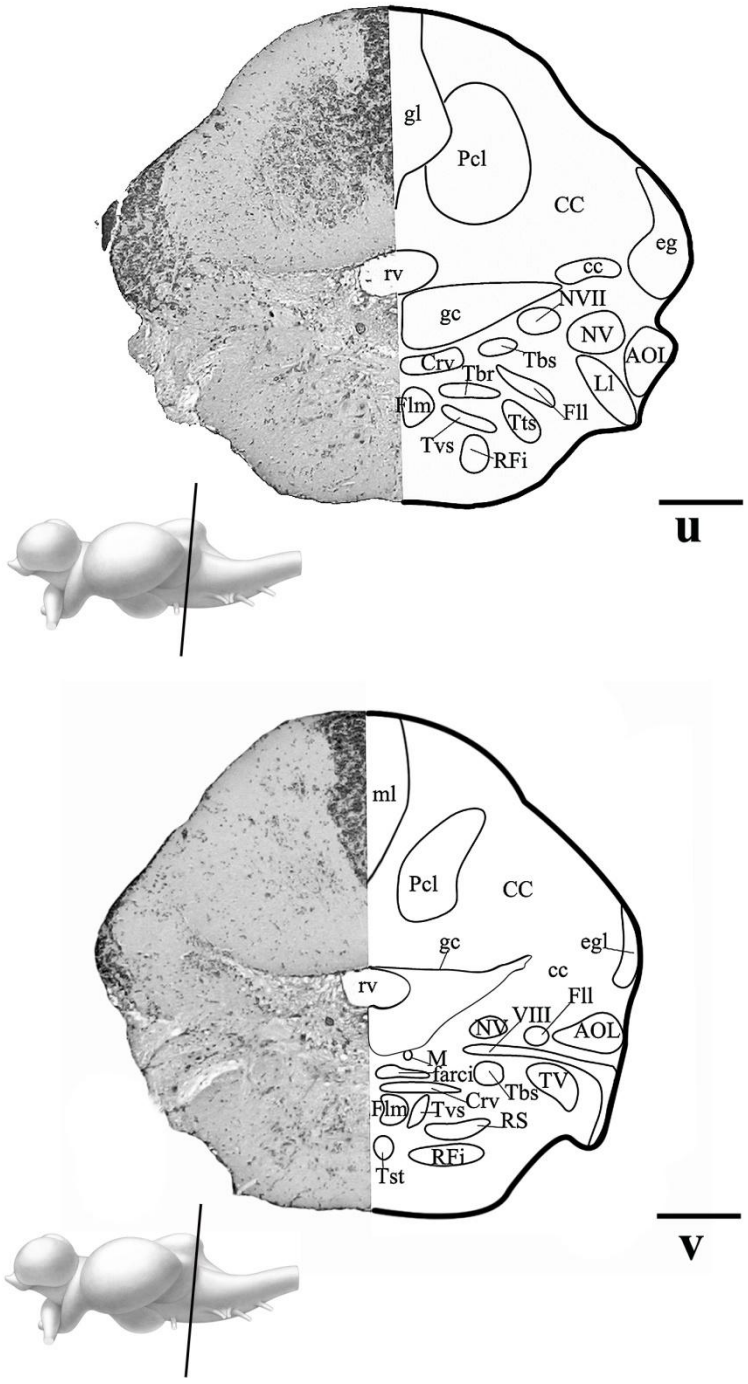


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

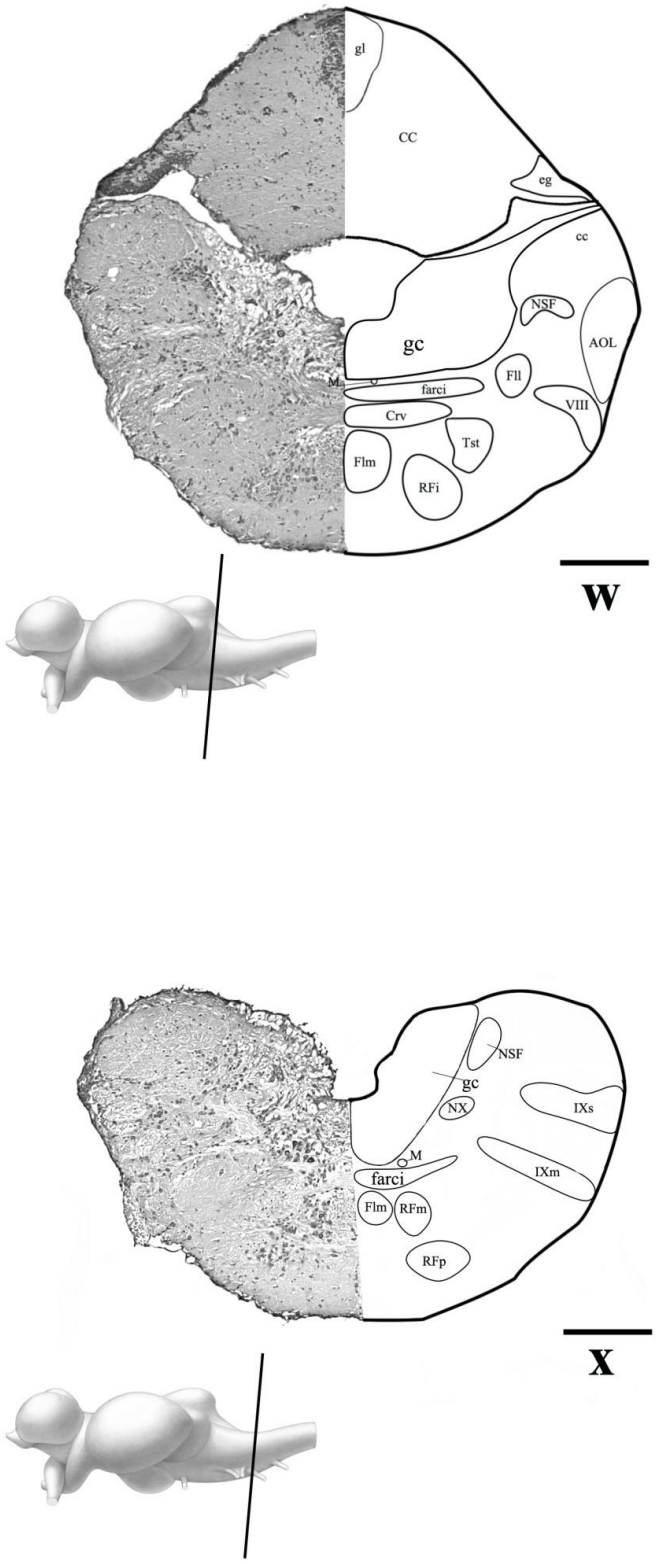


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

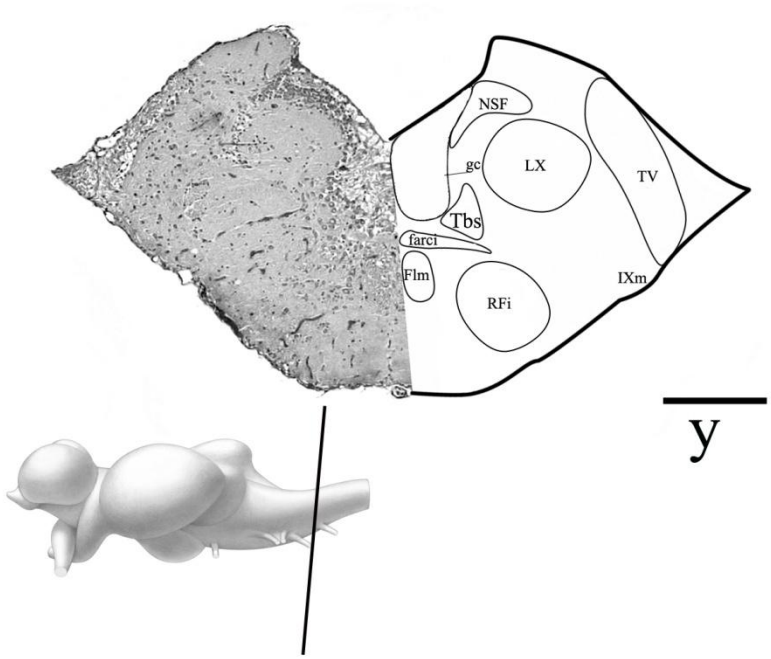


Fig 5. Cranio-caudal transverse sections of *N. furzeri* brain. a-j forebrain; k-r midbrain; s-y hindbrain. Scale bars a-y =200µm

Abbreviations

AC	*anterior commissure*
AOL	*octavolateral area*
AP	*pretectal area*
AT	*anterior thalamic nucleus*
Bc	*conjuntival brachium*
Can	*commissure ansulata*
cc	*cerebellar crista*
CC	*corpus of cerebellum*
CH	*horizontal commissure*
Ci	*interpeduncolar corpus*
CM	*mammillary corpus*
Crv	*ventral rhombencephalic commissure*
Cttd	*decussation of cerebello-tectal tract*
DcT	*dorso- central area of telencephalon*
DdT	*dorso-dorsal area of telencephalon*
DIL	*inferior diffuse lobe of hypothalamus*
DllT	*dorso-lateral area of lateral telencephalon*
DlT	*dorso-lateral area of telencephalon*
DmT	*dorso-medial area of telencephalon*
DOT	*dorsal optic tract*
DPT	*dorsal posterior thalamic nucleus*
DpT	*dorso- posterior area of telencephalon*
ecl	*external cell layer*
eg	*granular eminentiae*

EN	*entopeduncular nucleus*
ENa	*entopeduncolar area*
EW	*Edinger-Westphal nucleus*
farci	*internal arcuate fibers*
flb	*lateral forebrain bundle*
Fll	*lateral longitudinal fascicle*
Flm	*medial longitudinal fascicle*
FR	*retroflex fascicle*
gc	*central griseum*
Gl	*glomerular layer of olphactory bulbs*
gl	*granular layer of cerebellum*
H	*habenular nucleus*
HC	*caudal hypothalamus*
HD	*dorsal hypothalamus*
Hr	*hypothalamic recess*
HV	*ventral hypothalamus*
icl	*internal cell layer*
II	*nerve II*
III	*nerve III*
IV	*nerve IV*
IXm	*nerve IX (motor branch)*
IXs	*nerve IX (sensory branch)*
LV	*lateral nucleus of cerebellar valvula*
mes velum	*mesencephalic velum*
ml	*molecular layer of cerebellum*
MOT	*medial optic tract*

mv	*mesencephalic ventricle*
NC	*central nucleus*
NDTL	*diffuse nucleus of lateral torus*
NFlm	*nucleus of medial longitudinal fascicle*
NG	*glomerular nucleus*
NGa	*glomerular nucleus anterior part*
NI	*isthmic nucleus*
NIII	*nucleus of III nerve*
NIV	*nucleus of IV nerve*
NLlf	*nucleus of lateral longitudinal fascicle*
NR	*red nucleus*
NRP	*posterior recess nucleus*
NSF	*nucleus of solitary fascicle*
NT	*taenia nucleus*
NV	*nucleus of nerve V*
NX	*nerve X*
OB	*olphactory bulbs*
OT	*optic tectum*
PC	*posterior commessure*
Pc	*pretectal nucleus central part*
Pcl	*Purkinje cells layer*
Pd	*pretectal nucleus dorsal part*
PG	*preglomerular nucleus*
PGp	*preglomerular nucleus posterior part*
PGZ	*periglomerular grey zone*
Pi	*pretectal nucleus intermedial part*

PO	*paraventricolar organ*
Pp	*pretectal nucleus periventricular part*
PPa	*anterior preoptic nucleus*
PPm	*preoptic nucleus magnocellular portion*
PPp	*preoptic nucleus parvocellular portion*
Pv	*pretectal nucleus ventral part*
ra	*nucleus of raphe*
RFa	*anterior reticular formation*
RFi	*intermedial reticular formation*
RFm	*mesencephalic reticular formation*
RFp	*posterior reticular formation*
rv	*rhombencephalic ventricle*
SC	*suprachiasmatic nucleus*
Tbr	*bulbo-reticular tract*
Tbs	*bulbo-spinal tract*
Tbt	*tecto-bulbar tract*
Tc	*cerebellar tract*
Td	*dorsal tegmental nucleus*
TL	*longitudinal tori*
TN	*tuberal nucleus*
TNa	*tuberal nucleus anterior part*
TS	*semicircular tori*
Tts	*tecto-spinal tract*
TV	*tract of V nerve*
VC	*valvula of cerebellum*
VdT	*ventro-dorsal telencephalic area*

VII	*nerve VII*
VIII	*nerve VIII*
VlT	*ventro-lateral area of telencephalon*
VLT	*ventro-lateral thalamic nucleus*
VMT	*ventro-medial thalamic nucleus*
VOT	*ventral optic tract*
VpT	*ventro-posterior area of telencephalon*
VTN	*ventral thalamic nucleus*
VvT	*ventro-ventral area of telencephalon*

4.2 BDNF

4.2.1 ISH

ISH experiments were performed by using two probes, riboprobe and LNA probe. The patterns of expression of the two probes did not show differences in the anatomical distribution. Therefore, the present results report a unique neuroanatomical description of BDNF mRNA (Table 4).

Telencephalon

In the olphactory bulbs, some small and round cells in the primary olphactory nerve layer and in both external and internal layers were labeled (Fig 6a). The dorsal area of telencephalon showed a peculiar pattern of distribution of mRNA BDNF expressing neurons: numerous round small neurons, organized in layers in the most rostral part (Fig 6b), and less numerous and grouped in the most caudal part. Specifically, in the dorso-lateral and dorso-medial areas (Fig 6c) weakly stained neurons were seen. In the dorso-central telencephalic area (Fig 6c') an isolated group of neurons expressing BDNF were detected. In the ventral areas, mainly in the ventro-ventral area, few neurons were labeled. Along the surface of telencephalic ventricle the expression of BDNF was observed in small cells.

Diencephalon

In the most rostral part of the diencephalon, the preoptic area, the expression of BDNF mRNA was observed in neurons belonging to the parvocellular and magnocellular preoptic nuclei (Fig 6d) and to the suprachiasmatic nucleus (Fig 6d). In the epithalamus, the symmetric habenular nuclei appeared moderately labeled. In the thalamus, dorso-medially, strong labeling was observed in the anterior and ventral thalamic nuclei (Fig 6e) and in the intermediate one.

In the hypothalamus, small packed neurons, belonging to the dorsal and ventral hypothalamic nuclei (Fig 6f), were strongly labeled. Moreover, an intense staining was also observed in the anterior tuberal nucleus. More posteriorly, in the diffuse nucleus of inferior lobe grouped large neurons, expressing BDNF mRNA, were observed.

In the pretectum, strong positivity was observed in large neurons of the central nucleus (Fig 6e,g).

Along the ventricle, glial cells showed BDNF labeling.

Mesencephalon

Intense labeling of BDNF mRNA was detected in the optic tectum (OT), predominantly in the periventricular gray zone (PGZ) (Fig 7a), where numerous cells resulted strongly labeled, in the central white and grey zone (CZ) (Fig 7a), where sparse and small positive neurons with processes projecting towards the external layer. Finally, many small positive cells were also localized on the external layer of the superficial white and grey zone (SWGZ) (Fig 7a) of OT.

A slight staining was detected in the small and packed cells of longitudinal tori (Fig 7b). Intense BDNF mRNA expression was seen in the cells localized between the longitudinal tori and OT, (Fig 7b). Along the border of the glomerular nucleus (Fig 7c,d) many positive large neurons were detected, and in the horizontal commissure some scattered neurons (Fig 7c) were labeled. High positivity was seen in grouped neurons of semicircular tori (Fig 7e). Intensely labeled small grouped neurons were observed in the dorsal tegmental nucleus (Fig 7f). Intensely labeled neurons were detected in the nucleus of longitudinal fascicle, whereas some weak positivity was also detected in the fibers of longitudinal medial and lateral fascicles.

Large intensely labeled neurons showing evident cytoplasmatic processes were observed in the nucleus of III and V (Fig 7f) cranial nerves.

Rhombencephalon

In the cerebellum, the BDNF mRNA expression was observed in the Purkinje cells in the valvula (Fig 8a,b) and body of cerebellum (Fig 8c,d). Still in the body, cells of the granular layer and the radial glia at the uppermost area of the body showed BDNF mRNA expression (Fig 8c,d).

In the medulla oblongata, intense labeling was seen in neurons of the nucleus VII cranial nerve, and in small neurons of the nucleus of octavolateral area. Some positive fibers were seen in the secondary gustatory tract. Moreover, large

neurons belonging to the anterior, intermediate and posterior reticular formation showed high mRNA BDNF expression.

Along the rhombencephalic ventricle, some positive glial cells were detected.

Table 4*

BDNF *ISH*		
TELENCEPHALON		
olphactory bulbs	primary olfactory fiber layer	+
	internal and external cell layer	+
dorsal telencephalon	dorso-lateral	+
	dorso-medial	++
	dorso-central	+
ventral telencephalon	ventro-ventral	+
ventricle		+
DIENCEPHALON		
preoptic area	preoptic nuclei	++
	suprachiasmatic nucleus	+
epithalamus	habenular nuclei	+
thalamus	anterior thalamic nucleus	+++
	intermediate thalamic nucleus	++
	ventral thalamic nucleus	++
hypothalamus	dorsal and ventral hypothalamus	++
	anterior tuberal nucleus	++
	diffuse nucleus of inferior lobe	+
pretectum area	cortical nucleus	+++
ventricle		+
MESENCEPHALON		
optic tectum	PGZ	+++
	CZ	+
	SWGZ	++
longitudinal tori		+/-
tegmentum	glomerular nucleus	++
	horizontal commissure	+
	semicircular tori	+
	dorsal tegmental nucleus	++
	nucleus of longitudinal fascicle	+
	nucleus of III-V	+
RHOMBENCEPHALON		
cerebellum	valvula	++
	body	++
medulla oblongata	nucleus of VII	+
	nucleus of octavo-lateral area	++
	gustatory tract	+
	reticular formation (anterior, medial and posterior)	++
ventricle		+
* **+++ strong; ++ mild; + weak; +/- very weak**		

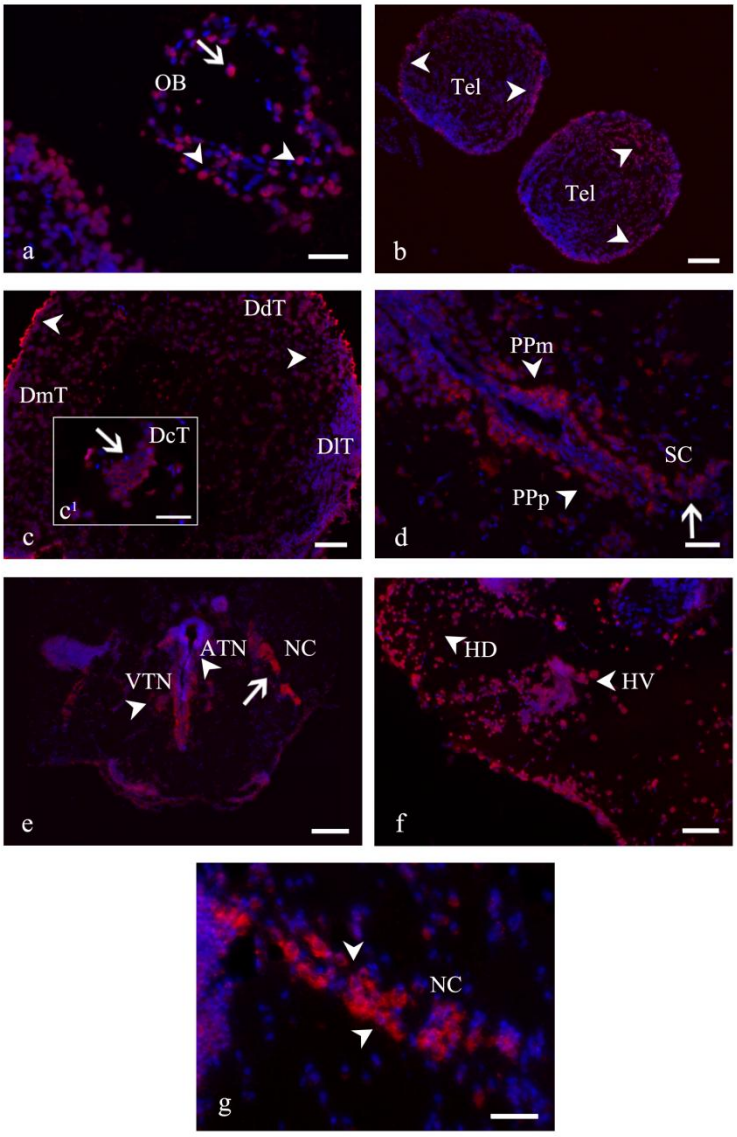


Fig 6. Expression of BDNF mRNA in the CNS. **Transverse forebrain sections. a.** BDNF mRNA expressed in external (arrowheads) and inner cells layers (arrows) of olphactory bulbs (OB). **b.** BDNF mRNA expressed in round and small neurons, organized in layers, (arrowheads) in the most rostral part of telencephalon (Tel). **c-c'.** BDNF mRNA expressed in the dorso-lateral (DlT) and dorso-medial (DmT) telencephalic areas (arrowheads) and in an isolated group of neurons in the dorso-central (DcT) telencephalic area (arrow). **d.** BDNF mRNA expressed in neurons of preoptic nuclei, magno- (PPm) and parvocellular (PPp) portions (arrowheads), and in neurons of suprachiasmatic nucleus (SC) (arrow). **e.** BDNF mRNA expressed in neurons of the anterior (ATN) and ventral thalamic (VTN) nuclei (arrowheads) and in the central nucleus (NC) of the pretectal area (arrow). **f.** BDNF mRNA expressed in small packed neurons, belonging to the dorsal (HD) and ventral (HV) hypothalamic nuclei (arrowheads). **g.** BDNF mRNA expressed in neurons of the central nucleus (NC) (arrowheads). Scale bars: **a-d** = 100 µm; **b-e** = 400 µm; **c-f** = 200 µm; **c'-g** = 50 µm.

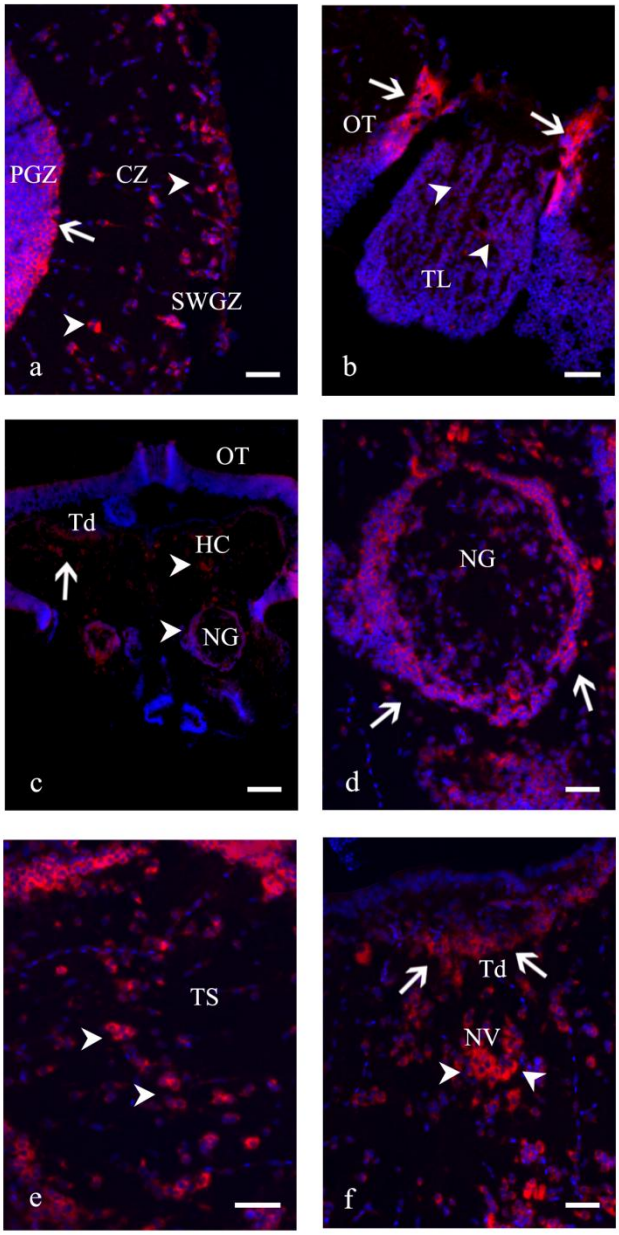


Fig 7. Expression of BDNF mRNA in the CNS. Transverse midbrain sections. a. BDNF mRNA expressed in the periventricular gray zone (PGZ) (arrow), in the central white (CZ) and grey zone and superficial white and grey zone (SWGZ) (arrowheads). **b.** BDNF mRNA in the small and packed cells of longitudinal tori (TL) (arrowheads) and in the cells and fibers localized between the longitudinal tori (TL) and optic tectum (OT) (arrows). **c.** overview of the expression of BDNF in the tegmentum: arrow indicates the expression in neurons of the dorsal tegmental nucleus (Td); arrowheads in neurons of the glomerular nucleus (NG) and horizontal commissure (CH). **d.** BDNF mRNA expressed in large neurons along the border of the glomerular nucleus (NG) (arrows). **e.** BDNF mRNA expressed in grouped neurons of the semicircular tori (TS) (arrowheads). **f.** BDNF mRNA expressed in neurons of the dorsal tegmental nucleus (Td) (arrows) and of the NV cranial nerve (arrowheads). Scale bars: **a-b-d-e-f** = 100 µm; **c** = 400 µm.

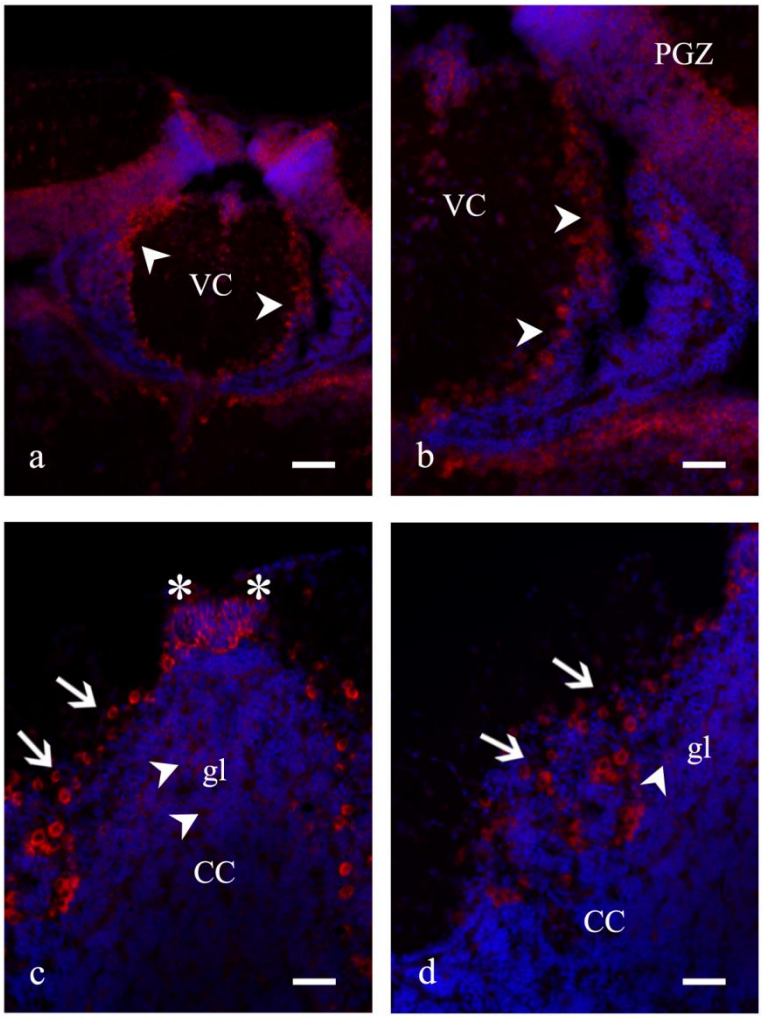


Fig 8. Expression of BDNF mRNA in the CNS. Transverse hindbrain sections. a. BDNF mRNA expressed in Purkinje cells (arrowheads) in the valvula of cerebellum (VC). **b.** High magnification of Purkinje cells in the valvula of cerebellum (VC) expressing mRNA BDNF (arrowheads). **c.** BDNF mRNA expressed in Purkinje cells (arrows) in the body of cerebellum (CC), in cells of the granular layer (gl) (arrowheads) and in radial glial cells (asterisks) of the body of cerebellum (CC). **d.** High magnification of Purkinje cells (arrows) in the body of cerebellum (CC) expressing BDNF mRNA and in few small cells of the glomerular layer (gl) (arrowhead). Scale bars: **a** = 200 µm; **b =** 100 µm; **c-d** = 50 µm.

4.2.2 IHC

IHC technique was carried out by using two antibodies, to compare whether the antigen distribution over the tissue was overlapped. Moreover, experiments with each antibody were performed either on paraffin and cryostatic serial sections, in order to exclude possible variations due to the fixation methods. The obtained results did not show differences in terms of neuronal distribution and intensity of immunoreactivity. Therefore, the neuroanatomical description of localization of BDNF protein refers to both antibodies, without any discrimination (Table 5).

Telencephalon

In the telencephalon, the BDNF immunoreactivity (IR) was detected in small cells and fibers spread over the olphactory bulbs (Fig 9a); particularly numerous immunoreactive fibers were grouped in the glomerular layer (Fig 9a).

In the telencephalic lobes, BDNF protein was distributed along the most superficial layer, the pial margin (Fig 9b), where dense glial cells were stained. In the dorso-medial, dorso-central and dorso-lateral (Fig 9b) areas of the telencephalon numerous small neurons resulted intensely positive to BDNF protein. Moreover, in the dorso-central area of central telencephalon, grouped neurons resulted well stained. Weak immunoreactive fibers were observed in the ventrodorsal and ventro-lateral areas of ventral telencephalon.

Diencephalon

In the diencephalon, BDNF immunoreactive neurons were seen in the entopeduncolar nucleus (Fig 9c), and in both magnocellular and parvocellular portion of the preoptic nucleus (Fig 9c,d), many neurons were immunoreactive to BDNF. Many positive fibers decurring in the medial and lateral forebrain bundles were observed. In the epithalamus, packed cells and fibers of the habenular nuclei (Fig 9d) were seen BDNF positive. In the thalamus, moderate IR was detected in neurons of the anterior and ventral (Fig 9d) thalamic nuclei. BDNF IR was also observed in small neurons of the paraventricolar organs. Most posteriorly, strong BDNF IR was seen in fibers and neurons of the central (Fig 9d) and preglomerular nuclei. In the hypothalamus, immunoreactive fibers

were seen in the posterior recess nucleus, and scattered neurons were present in the diffuse nucleus of inferior lobe (Fig 9e).

Mesencephalon

In the OT, many small cells resulted positive to BDNF protein were observe in the PGZ (Fig 10a) and numerous fibers and few neurons were positive in the layers of CZ and SWGZ (Fig 10a). In the longitudinal tori (Fig 10b) a BDNF fiber mash, intensely stained, was observed. In the glomerular nucleus BDNF IR was seen in numerous scattered fibers of the central part of nucleus and in some neurons localized at its dorsal and ventral margin (Fig 10c). Moreover, few positive neurons were detected in the horizontal commissure. Intense staining was observed in the neurons of semicircular tori and dorsal tegmental nucleus (Fig 10d). Glial cells localized along the mesencephalic ventricle resulted also positive to BDNF.

Rhombencephalon

In the body of cerebellum, BDNF positivity was observed in the cells of the molecular layer and in numerous Purkinje cells. Posterior, in the apical part of cerebellum body, strong staining was seen in glial cells. In the medulla oblongata, intense BDNF staining was detected in neurons and fibers of the solitary fascicle nucleus (Fig 10e,f). Moreover, positivity was seen in dense fibers of octavolateral area (Fig 10e) and in the internal arcuate fibers, and in neurons of the anterior reticular formation (Fig 10e).

Table 5*

BDNF *IHC*		
TELENCEPHALON		
olphactory bulbs	glomerular layer	++
dorsal telencephalon	dorso-dorsal	++
	dorso-lateral	++
	dorso-medial	++
	dorsocentral	++
ventral telencephalon	ventro-dorsal	+
	ventro-lateral	+
DIENCEPHALON		
entopeduncolar nucleus		++
preoptic area	preoptic nuclei	++
forebrain bundle	medial and lateral	+
epithalamus	habenular nuclei	+
thalamus	anterior thalamic nucleus	+
	ventral thalamic nucleus	++
	paraventricolar organ	+
	cortical nucleus	++
	preglomerular nucleus	++
hypothalamus	posterior recess nucleus	+
	diffuse nucleus of inferior lobe	+
pretectum area	cortical nucleus	+++
ventricle		+
MESENCEPHALON		
optic tectum	PGZ	+
	CZ	+/-
	SWGZ	+/-
longitudinal tori		+
tegmentum	glomerular nucleus	++
	horizontal commissure	+/-
	semicircular tori	
	dorsal tegmental nucleus	
ventricle		+
RHOMBENCEPHALON		
cerebellum	body	++
medulla oblongata	nucleus of solitary fascicle	++
	nucleus of octavo-lateral area	++
	internal arcuate fibers	+
	reticular formation (anterior)	++
***** **+++ strong; ++ mild; + weak; +/- very weak**		

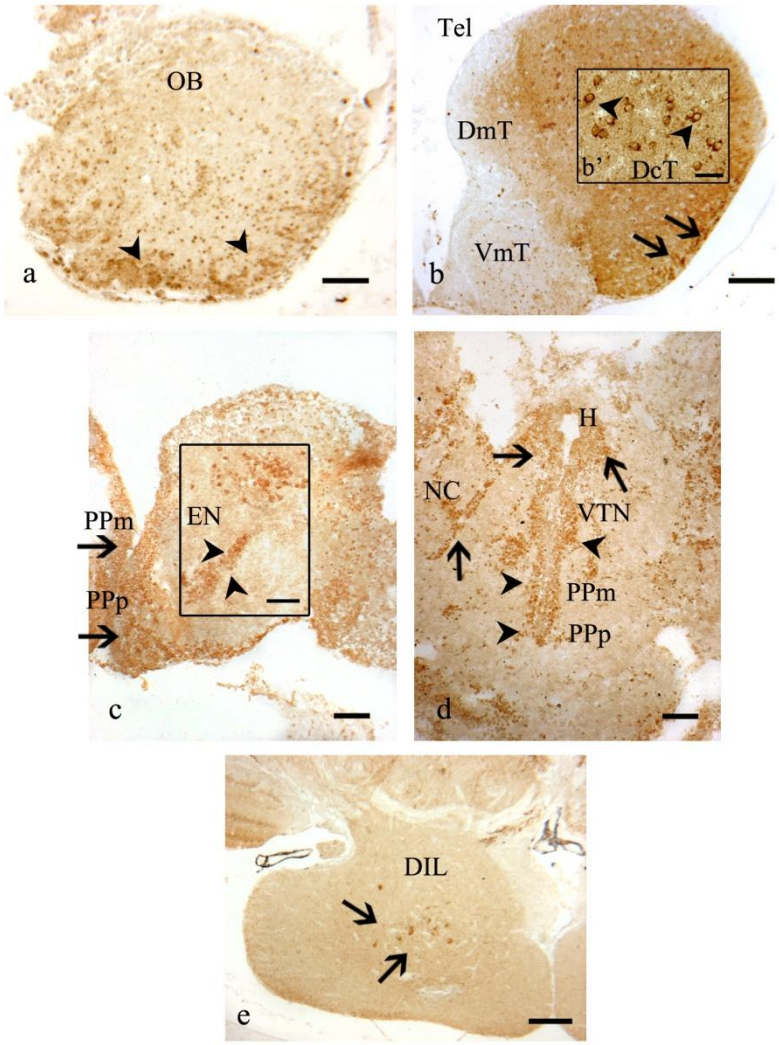


Fig 9. Distribution of BDNF antigen in the CNS. Transverse forebrain sections. a. BDNF protein distributed in small cells and fibers spread over the olphactory bulbs (OB), particularly numerous grouped immunoreactive fibers in the glomerular layer (arrowheads). **b-b'.** BDNF protein distributed in small neurons of dorso-medial (DmT) and dorso-central (DcT) telencephalic areas (arrowheads), and in glial cells along the pial telencephalic margin (arrows). **c.** BDNF protein distributed in neurons of the entopeduncolar nucleus (EN) (arrowheads) and in neurons of the preoptic nuclei, magno- (PPm) and parvocellular (PPp) portions (arrows). **d.** BDNF protein distributed in neurons of the preoptic nuclei, magno- (PmN) and parvocellular (PpN) portions, and ventral thalamic nucleus (arrowheads), in packed cells and fibers of the habenular nuclei (H) and in neurons of the central nucleus (NC) (arrows). **e.** BDNF protein distributed in scattered neurons of the diffuse nucleus of inferior lobe of hypothalamus (DIL) (arrows). Scale bars: **a-e** = 50 µm; **b- c'** = 100 µm; **b'** = 25 µm; **c-d** = 200 µm.

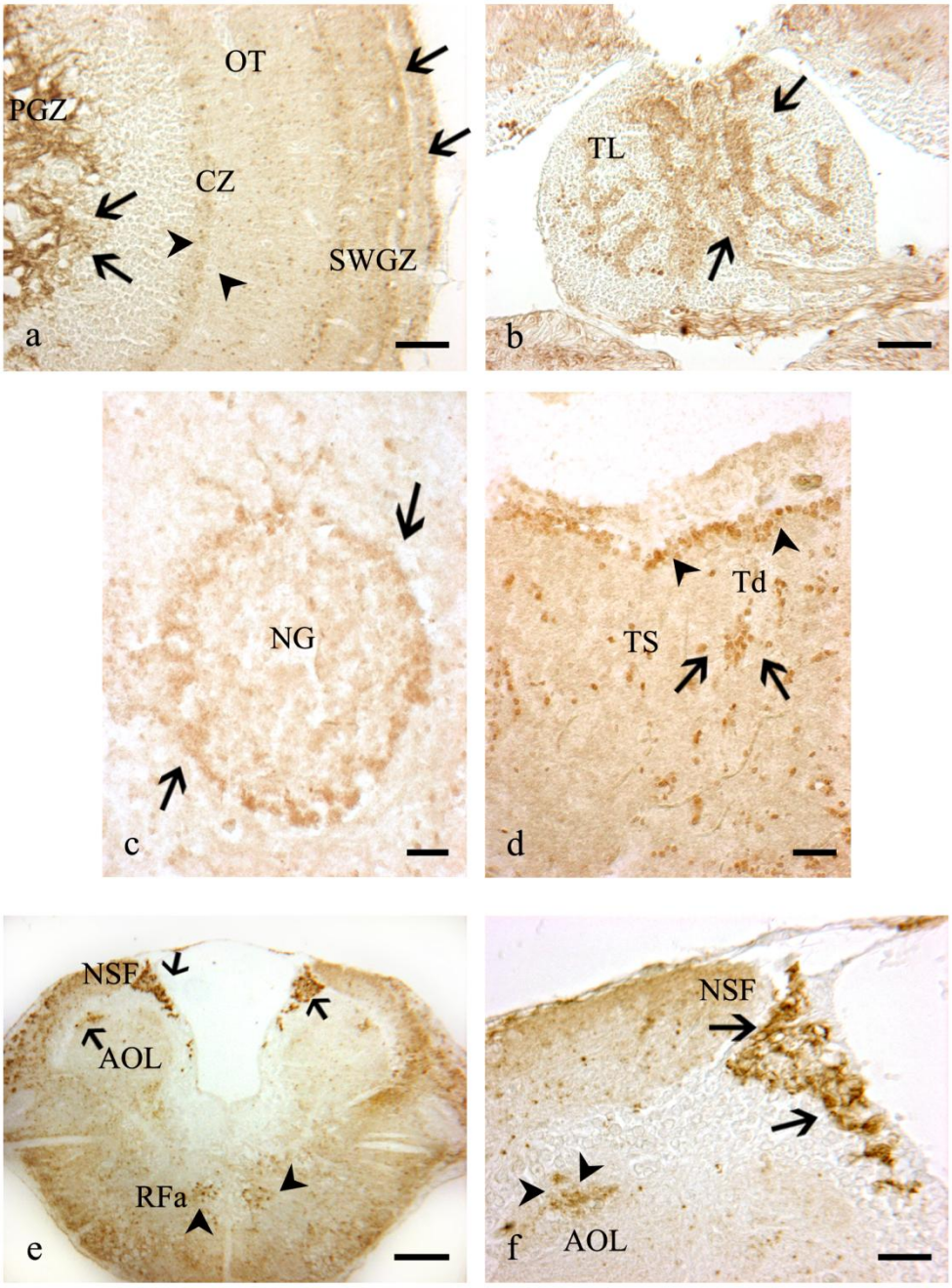


Fig 10. Distribution of BDNF antigen in the CNS. Transverse midbrain (a-d) and hindbrain (e-f) sections. a. BDNF protein distributed in small cells and fibers of the periventricular zone (PGZ) (arrows), in numerous fibers and few neurons of the layers of central zone CZ (arrowheads) and superficial white and grey zone (SWGZ) (arrows) in the OT. **b.** BDNF protein distributed in fiber mash of the longitudinal tori (TL) (arrows). **c.** BDNF protein distributed in neurons along the dorsal and ventral margins of the glomerular nucleus (NG) (arrows). **d.** BDNF protein distributed in neurons of the semicircular tori (TS) (arrows) and dorsal tegmental nucleus (Td) (arrowheads). **e.** BDNF protein distributed in dense fibers of octavolateral area (AOL) (arrow), in neurons and fibers of the solitary fascicle nucleus (NSF) (arrows) and in neurons of the anterior reticular formation (RFa) (arrowheads). **f.** High magnification of BDNF protein distributed in neurons and fibers of the solitary fascicle nucleus (NFS) (arrows) and in fibers of the octavolateral area (AOL) (arrowheads). Scale bars: **a-b-f** = 50 µm; **c-d** = 25 µm; **e** = 100 µm.

4.3 NGF

4.3.1 ISH (Table 6)

Telencephalon

NGF mRNA was expressed in few cells of the internal cellular layer and glomerular layer of the olphactory bulbs. Labeled small and round cells were dispersed among the fibers of the olphactory tract. In the telencephalon, neurons were diffusely labeled in the dorsal areas, predominantly in the dorso-central area (Fig 11a,b). Additionally, NGF probe signal was detected in the ventral area of telencephalon. Particularly, in the ventro-lateral and ventro-ventral areas of ventral telencephalon intensely stained cells were seen. In the most posterior region of telencephalon, some labeled neurons, belonging to the taenia nucleus, were observed (Fig 11c).

Diencephalon

The nucleus preoptic, both parvo- and magnocellular portion, resulted intensely labeled.

In the epithalamus, packed and small cells located in the ventral part of the habenular nuclei expressed NGF mRNA.

In the most anterior region of thalamus, few and small neurons with some evident cytoplasmatic processes were seen in the entopeduncular nucleus. Larger few neurons were detected in the anterior and more numerous in ventral thalamic nucleus (Fig 11d).

In the hypothalamus, the diffuse nucleus of inferior lobe showed a peculiar pattern characterized by a continuous line of labeled cells along the margin (Fig 11e). These cells were small and round in shape. Moreover, few large neurons (Fig 11e,f) with evident processess were also labeled in the inner part of the inferior diffuse lobe.

In the most caudal part of diencephalon, positive cells were observed in the cortical nucleus.

Mesencephalon

The expression of NGF mRNA was observed in the OT, particularly in the PGZ, in the layers of the SWGZ where small cells resulted labeled (Fig 12a) and in positive neurons of the CZ (Fig 12a). In the tegmentum of mesencephalon, the glomerular nucleus showed intense staining. In the posterior portion of the nucleus, few and large positive neurons were localized along the ventral margin. More caudally, numerous labeled cells were observed in the dorsal tegmental nucleus (Fig 12b). Diffuse positive neurons expressing mRNA NGF were detected in the semicircular tori (Fig 12b). Fibers belonging to the longitudinal lateral and medial fascicles were also labeled. NGF transcript was detected in the neurons of the nucleus of V nerve.

Rhombencephalon

In the rhombencephalon, NGF mRNA was expressed in some Purkinje cells of the valvula and of the body (Fig 12c,d) of cerebellum. In the latter, at the upper portion radial glial cells (Fig 12c) showed an intense labeling. Additionally, an intense labeling was observed in the cells of granular eminentiae. In medulla oblongata, positive cells organized in a row were observed below the glial cells bordering the dorsal margin of the rhombencephalic ventricle (Fig 12e, f). The neurons of anterior (Fig 12f), medial and posterior reticular formation resulted clearly labeled. The expression of NGF was localized also in the fibers of gustatory tract, and in few neurons of the sensory portions of the nucleus of VII nerve (Fig 12e,f) and in few neurons of the octavolateral area, where the labeling was weakly.

Table 6*

NGF *ISH*		
TELENCEPHALON		
olphactory bulbs	internal cell layer	+
	glomerular layer	+
	olphactory tract	+/-
dorsal telencephalon	dorso-dorsal	+
	dorso-lateral	++
	dorso-medial	+
	dorso-central	++
ventral telencephalon	ventro-lateral	+
	ventro-ventral	+
taenia nucleus		+
DIENCEPHALON		
preoptic area	preoptic nuclei	++
epithalamus	habenular nuclei	+
thalamus	entopeduncular nucleus	+
	anterior thalamic nucleus	+
	ventral thalamic nucleus	++
hypothalamus	diffuse inferior lobe	++
pretectum area	cortical nucleus	++
MESENCEPHALON		
optic tectum	PGZ	++
	CZ	+
	SWGZ	+
tegmentum	glomerular nucleus	++
	dorsal tegmental nucleus	+
	semicircular tori	+
	longitudinal lateral and medial fascicles	+
	nucleus of V	+
RHOMBENCEPHALON		
cerebellum	valvula	+
	body	+
	granular eminentiae	+
	reticular formation (anterior, medial, posterior)	++
	gustatory tract	+
	nucleus of octavolateral area	+/-
ventricle		++

*** +++ strong; ++ mild; + weak; +/- very weak**

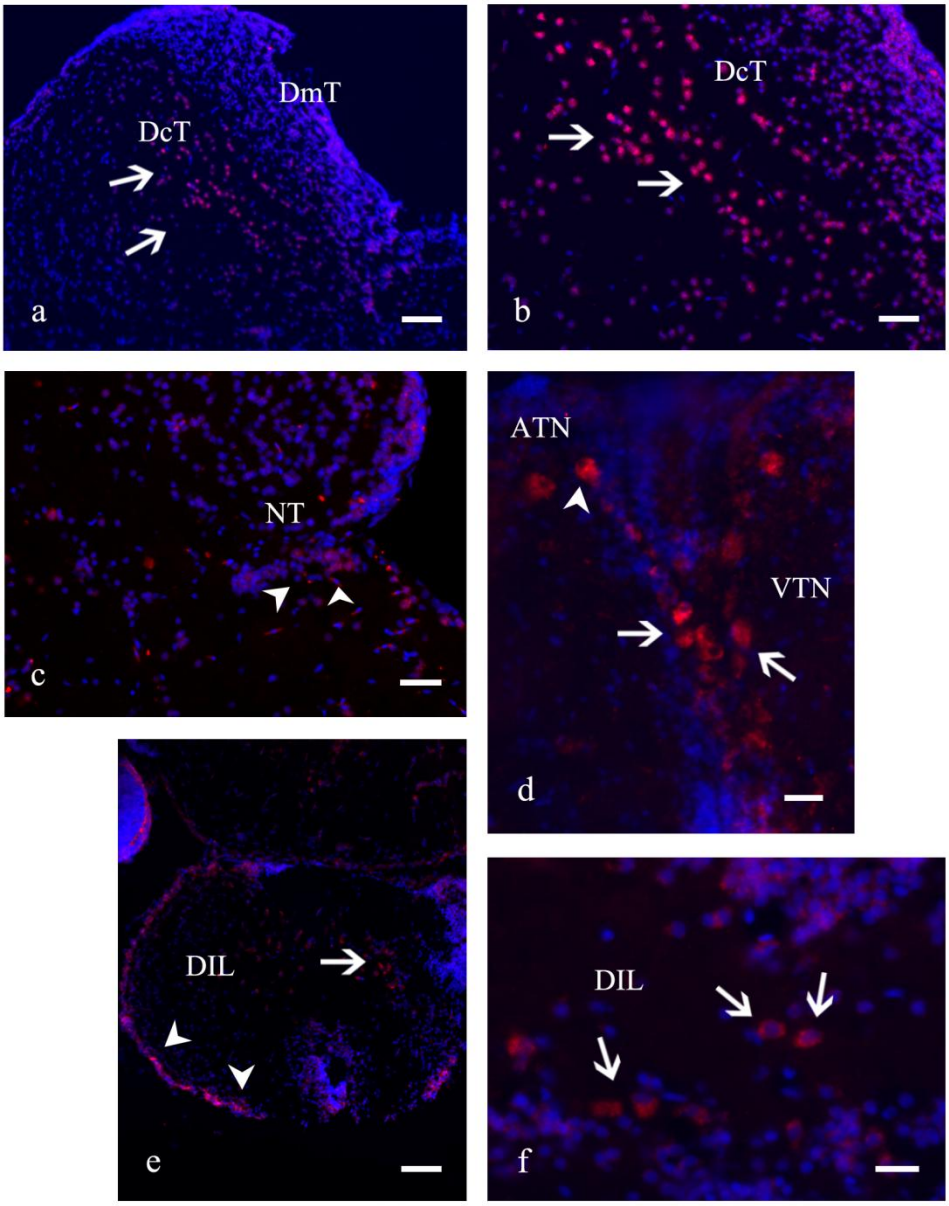


Fig 11. Expression of NGF mRNA in the CNS. Transverse forebrain sections. a-b. mRNA NGF expressed in diffuse neurons of the dorso-central (DcT) telencephalic area (arrows) at low (a) and high (b) magnification. **c.** NGF mRNA expressed in labeled neurons of the taenia nucleus (NT) (arrowheads). **d.** NGF mRNA expressed in neurons of the anterior (ATN) (arrowheads) and ventral thalamic nucleus (VTN) (arrows). **e.** NGF mRNA expressed in cells along the margin of the diffuse nucleus of inferior hypothalamus (DIL) (arrowheads) and in neurons and their processes of the diffuse nucleus of inferior hypothalamus (DIL) (arrows). **f.** NGF mRNA expressed in neurons and their processes of the diffuse nucleus of inferior hypothalamus (DIL) (arrows). Scale bars: **a-e** = 200 µm; **b-c** = 100 µm; **d-f** = 50 µm.

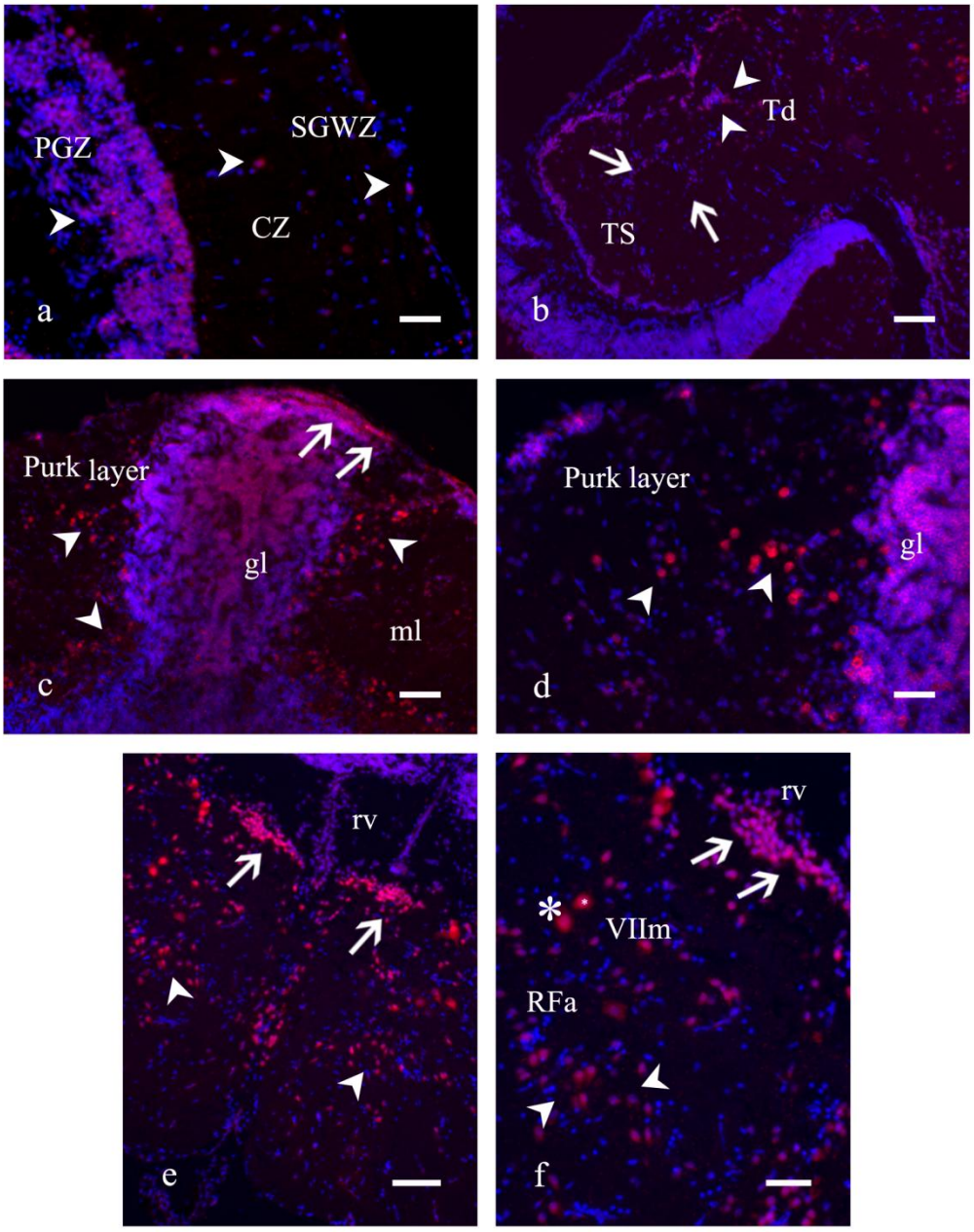


Fig 12. Expression of NGF mRNA in the CNS. Transverse midbrain (a-b) and hindbrain (c-f) sections. a. mRNA NGF expressed in the cells of the periventricular grey zone (PGZ) (arrowheads), in few neurons of central zone CZ) (arrowheads) and superficial white and grey zone (SWGZ) (arrowheads) of OT. **b.** NGF mRNA expressed in neurons of the semicircular tori (TS) (arrows) and in the dorsal tegmental nucleus (Td) (arrowheads). **c.** mRNA NGF expressed in Purkinje cells (arrowheads) of the body of cerebellum and in radial glial cells (arrows) at the upper portion of the body. **d.** High magnification of NGF mRNA expressed in Purkinje cells (arrowheads) of the body of cerebellum. **e,f.** NGF mRNA expressed in glial cells (arrows) bordering the dorsal margin of the rhomboencephalic ventricle (rv), in neurons of the VII cranial nerve, motor branch (asterisk), and in neurons of the anterior reticular formation (RFa) (arrowheads) at low (e) and high (f) magnification. Scale bars: **a-d-f** = 100 µm; **b-c-e** = 20 µm.

4.3.2 IHC **(Table 7)**

Telencephalon

NGF IR was appreciated in few positive small and round cells dispersed in the external cell layer of the olphactory bulbs. Very few neurons and fibers were positive in the glomerular layer. Additionally, immunopositive fibers of the olphactory nerve were detected.

In the telencephalic hemispheres, NGF protein distribution was seen in round or oval shaped neurons, often showing a thin short process, of the dorso-lateral, dorso-central and dorso-medial areas (Fig 13a). Small neurons of the ventro-lateral and ventro-ventral areas of telencephalon were weakly stained and rather numerous. In the ventro-posterior telencephalon, grouped neurons were clearly immunopositive to NGF (Fig 13a,b). Along the ventricle, sparse small cells, morphologically identifiable as glial cells, were frequently detected (Fig 13a,b).

Diencephalon

In the most rostral part of diencephalon, neurons of the entopeduncolar nucleus were moderately immunostained. In the epithalamus, small positive cells of the habenular nuclei resulted positive. In the thalamus, NGF IR was observed in neurons of the ventral thalamic nucleus. In the hypothalamus, some ovoidal positive neurons were localized in the diffuse nucleus of the inferior lobe. Glial cells with their processes were stained along the diencephalic ventricle. Most posteriorly, neurons belonging to the pretectal nucleus and respective processes were immunopositive.

Mesencephalon

In the glomerular nucleus, some NGF positive neurons were distributed along the ventral boundary (Fig 13c,d). Few neurons of the medial longitudinal fascicle nucleus were also immunostained.

Rhombencephalon

In the body of cerebellum numerous Purkinje cells resulted NGF immunoreactive (Fig 13e,f). In the medulla oblongata, many neurons intensely stained were localized in neurons of medial and posterior reticular formation and, moreover, in the nucleus of VIII nerve.

Table 7*

NGF IHC		
TELENCEPHALON		
olphactory bulbs	external cell layer	+/-
	glomerular layer	+/-
	olphactory nerve	+
dorsal telencephalon	dorso- lateral	++
	dorso- medial	+
	dorso-central	++
ventral telencephalon	ventro-lateral	+
	ventro-ventral	+
ventricle		+
DIENCEPHALON		
entopeduncolar nucleus		+
epithalamus	habenular nuclei	+
thalamus	ventral thalamic nucleus	++
hypothalamus	diffuse nucleus of inferior lobe	++
pretectum area	pretectal nucleus	++
ventricle		+
MESENCEPHALON		
tegmentum	glomerular nucleus	++
	nucleus of longitudinal medial fascicle	+
RHOMBENCEPHALON		
cerebellum	body	+
medulla oblongata	reticular formation (medial, posterior)	++
	nucleus of VIII	++
* **+++ strong; ++ mild; + weak; +/- very weak**		

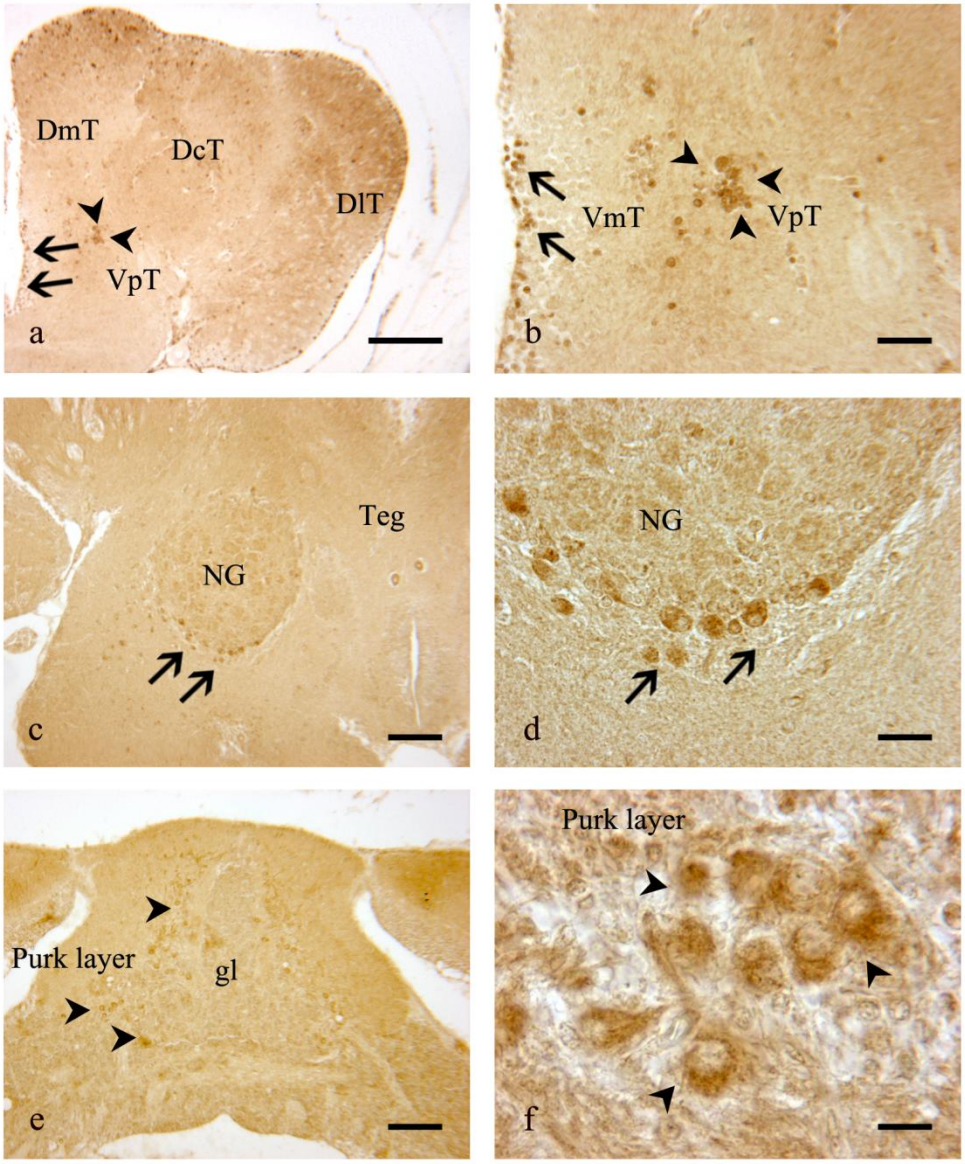


Fig 13. Distribution of NGF antigen in the CNS. Transverse forebrain (a-b), midbrain (c-d), hindbrain (e-f) sections. a,b. NGF protein distributed in a group of neurons in the posterior dorsal telencephalon (DpT) (arrowheads), and in glial cells along the ventricle (arrows) at low (a) and high (b) magnification. **c,d.** NGF protein distributed in neurons along the ventral boundary (arrows) of the glomerular nucleus (NG) at low (c) and high (d) magnification. **e,f.** NGF protein distributed in Purkinje cells (arrowheads) of the body of cerebellum (CC) at low (e) and high (f) magnification. Scale bars: **a-c-e** = 100 μm; **b-d** = 25 μm; **f** = 10 μm.

4.4 NT-3

4.4.1 ISH

Any neurons or cellular populations expressed NT-3 mRNA.

4.4.2 IHC **(Table 8)**

NT-3 protein distribution was observed only in few cellular elements of the diencephalic region. Particularly, in the caudal hypothalamus single pear-shaped neurons sending cytoplasmic prolongaments toward the inner regions were observed. Along the diencephalic ventricle only few ependimocytes resulted positive to NT-3.

Table 8*

NT-3 *IHC*		
DIENCEPHALON		
hypothalamus	caudal hypothalamus	+
ventricle		+
* **+++ strong; ++ mild; + weak; +/- very weak**		

4.5 NT-4

4.5.1 ISH (Table 9)

Telencephalon

In the olphactory bulbs, few and sparse positive cells localized in the internal cell layers were weakly stained (Fig 14a), whereas either in medial and lateral olphactory tract, the fibers labeling was intense (Fig 14a). The telencephalon showed a strong staining, mainly evident in the neurons of the dorsal areas, specifically in the dorso-lateral, dorso-medial and dorso-dorsal (Fig 14b) areas. In the dorso-central area, a group of neurons resulted clearly labeled, whereas less intense staining was observed in the ventral telencephalic areas. In the posterior telencephalon, along the ventricle a group of cells, morphologically identifiable as glial cells, showed intense labeling (Fig 14c). In the most posterior regions of the telencephalon, some NT-4 mRNA neurons belonging to the taenia nucleus were labeled.

Diencephalon

A strong probe signal was observed in few neurons of the preoptic nucleus, stronger in parvocellular than in magnocellular portion and in the processes of lateral forebrain bundle. Few, well labeled neurons were observed in the anterior and ventral thalamic nuclei (Fig 14d). In the pretectal area, neurons of the central nucleus expressed NT-4 mRNA (Fig 14e). In the optic nerve some positive glial cells were detected.

Mesencephalon

The NT4 mRNA labeling was detected in the OT, mainly in numerous positive neuronal elements of the PGZ, in few dispersed neurons of the CZ (Fig 14f) with processes projecting toward the marginal layer and also in numerous large neurons of the SWGZ (Fig 14f). In the longitudinal tori labeled neurons were observed. Intensely labeling was detected in large neurons of the nucleus V nerve.

Rhombencephalon

NT-4 mRNA presence was observed in some Purkinje cells localized in the cerebellum body (Fig 14g) and in some neurons of the caudal cerebellar lobe. Some positive neurons belonging to the granular eminentiae expressed NT-4 mRNA. Intense labeling was observed in the line bordering the margin between the cerebellum body and the most posterior part of optic tectum (Fig 14h). The labeling for NT-4 mRNA was rather diffuse in the neurons belonging to the nucleus of octavolateral area.

Table 9*

NT-4 *ISH*		
TELENCEPHALON		
olphactory bulbs	internal cell layer	+/-
	olphactory tract	++
dorsal telencephalon	dorso-dorsal	+++
	dorso-lateral	++
	dorso-medial	+
	dorso-central	+
ventral telencephalon	ventro-lateral	+/-
	ventro-ventral	+/-
taenia nucleus		+
DIENCEPHALON		
preoptic area	preoptic nuclei	++
thalamus	lateral forebrain bundle	+
	anterior thalamic nucleus	+/-
	ventral thalamic nucleus	+
	cortical nucleus	+
optic nerve		+
MESENCEPHALON		
optic tectum	PGZ	++
	CZ	+/-
	SWGZ	++
longitudinal tori		+
tegmentum	nucleus of V	++
RHOMBENCEPHALON		
cerebellum	body	+
	caudal lobe	+
	granular eminentiae	+
medulla oblongata	nucleus of octavolateral area	++
* **+++ strong; ++ mild; + weak; +/- very weak**		

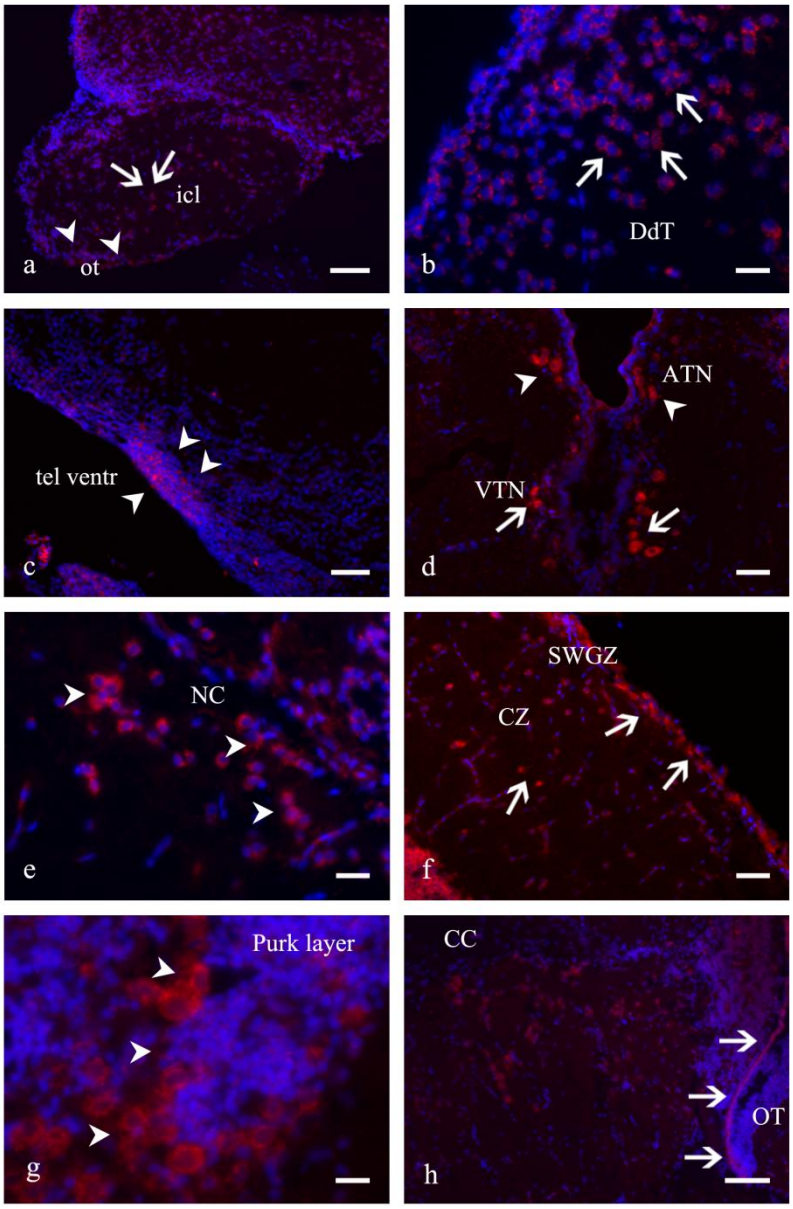


Fig 14. Expression of NT-4 mRNA in the CNS. Transverse forebrain (a-e), midbrain (f) and hindbrain (g-h) sections. a. NT-4 mRNA expressed in the cells of internal cell layers (icl) (arrows) of olphactory bulbs and in fibers (arrowheads) of the olphactory tract (ot). **b.** NT-4 mRNA expressed in neurons of the dorso-dorsal area (arrows) of telencephalon (DdT). **c.** NT-4 mRNA expressed in the glial cells (arrowheads) along the telencephalic ventricle (tel ventr) in the posterior telencephalon. **d.** NT-4 mRNA expressed in few neurons of the anterior thalamic nucleus (ATN) (arrowheads) and ventral thalamic nucleus (VTN) (arrows). **e.** NT-4 mRNA expressed in neurons of the central nucleus (NC) (arrowheads). **f.** NT-4 mRNA expressed in dispersed neurons with processes of the central zone (CZ) (arrows), and also in numerous large neurons of the superficial white and grey zone (SWGZ) (arrows) of the optic tectum (OT). **g.** NT-4 mRNA expressed in Purkinje cells (arrowheads) of the body of cerebellum. **h.** NT-4 mRNA expressed in cells (arrows) of the line between the cerebellum body and the most posterior part of optic tectum (OT). Scale bars: **a** = 200 µm; **b-d-e** = 50 µm; **c-f-h** = 100 µm; **g** = 30 µm.

4.5.2 IHC **(Table 10)**

Telencephalon

In the olphactory bulbs, IR to NT-4 was observed in few cells of the inner cell layer and in many cells of the glomerular layer (Fig 15a,b).

The distribution pattern of NT-4 in the telencephalic hemispheres was characterized by numerous and intensely stained neurons and fibers in the dorso-lateral (Fig 15b,c) areas. In the dorsal area of posterior telencephalon, the staining was detected in relatively large neurons.

Diencephalon

In the most anterior diencephalic region, NT-4 IR was present in the neurons and fibers of the entopeduncolar nucleus. In the thalamus, particularly in the preoptic area, immunopositivity was seen in many fibers and scarce neurons of the suprachiasmatic nucleus and immunoreactive neurons were detected in the preoptic nuclei, either in magno- and parvo-cellular portions.

In the ventral part of thalamus, numerous and strongly stained neurons were seen in the anterior tuberal nucleus. Still in the thalamus, fibers of the lateral forebrain fascicle and fibers of the ventral and dorsal optic tract were strongly immunoreactive to NT-4. In the hypothalamus, positive neurons and fibers were observed in the dorsal hypothalamus, whereas in other hypothalamic regions only positive neuronal populations were detected. Particularly, intense positive staining was seen in the neurons of the nucleus of lateral torus, in the nucleus of posterior recess and in the diffuse nucleus of inferior lobe of hypothalamus (Fig 15e). Moreover, neurons of the pretectal nucleus were immunopositive. In the optic nerve, few cells and many fibers showed intense staining. Along the ventricle, some small and round neurons resulted immunopositive.

Mesencephalon

NT-4 immunoreactivity was mainly localized in the OT, where numerous positive fibers were observed in layers of the CZ and in layers of the SWGZ. Immunopositivity was seen in scarce fibers of the longitudinal tori. In the mesencephalic tegmentum, many fibers and neurons of the preglomerular nucleus, resulted NT-4 immunoreactive. The glomerular nucleus (Fig 15d)

showed immunopositivity in frequent neurons distributed along the border of nucleus and in the fibers localized in the most central part. Also, in the horizontal commissure some neurons and fibers were seen positive. In the nucleus of the medial and lateral (Fig 15e,f) longitudinal fascicles many neurons were detected. More posterior, strong NT-4 positivity was seen in the neurons and fibers belonging to the semicircular tori and in neurons of the dorsal tegmental nucleus.

Rhombencephalon

The pattern of NT-4 protein distribution throughout the rhombencephalic region was characterized by few positive Purkinje cells in the valvula and in the body of cerebellum (Fig 15g). In the medulla oblongata, IR was detected in scattered neurons of the nucleus of solitary fascicle. Intensely positive neurons and fibers were distributed in the nucleus of octavolateral area and strong immunopositivity was detected in the fibers of the VIII nerve.

Finally, neurons of the anterior and intermedial reticular formation (Fig 15h) showed intense NT-4 immunoreactivity.

Table 10*

NT-4 *IHC*		
TELENCEPHALON		
olphactory bulbs	inner cell layer	+/-
	glomerular layer	++
dorsal telencephalon	dorso-lateral	+
	dorso-posterior	+
DIENCEPHALON		
entopeduncolar nucleus		+
preoptic area	suprachiasmatic nucleus	+
	preoptic nucleus	+
thalamus	anterior tuberal nucleus	++
	lateral forebrain fascicle	++
	dorsal and ventral optic tract	++
	dorsal hypothalamus	++
	nucleus of lateral torus	+++
	nucleus of posterior recess	++
	diffuse inferior lobe	++
pretectum area	pretectal nucleus	++
optic nerve		+
ventricle		+
MESENCEPHALON		
optic tectum	CZ	++
	SWGZ	++
longitudinal tori		+/-
tegmentum	preglomerular nucleus	++
	glomerular nucleus	++
	horizontal commissure	+
	nucleus of longitudinal fascicle	++
	semicircular tori	++
	dorsal tegmental nucleus	++
RHOMBENCEPHALON		
cerebellum	valvula	+/-
	body	+/-
medulla oblongata	nucleus of solitary fascicle	++
	nucleus of octavolateral area	+
	nucleus of VIII	++
	reticular formation (anterior, medial)	++
* **+++ strong; ++ mild; + weak; +/- very weak**		

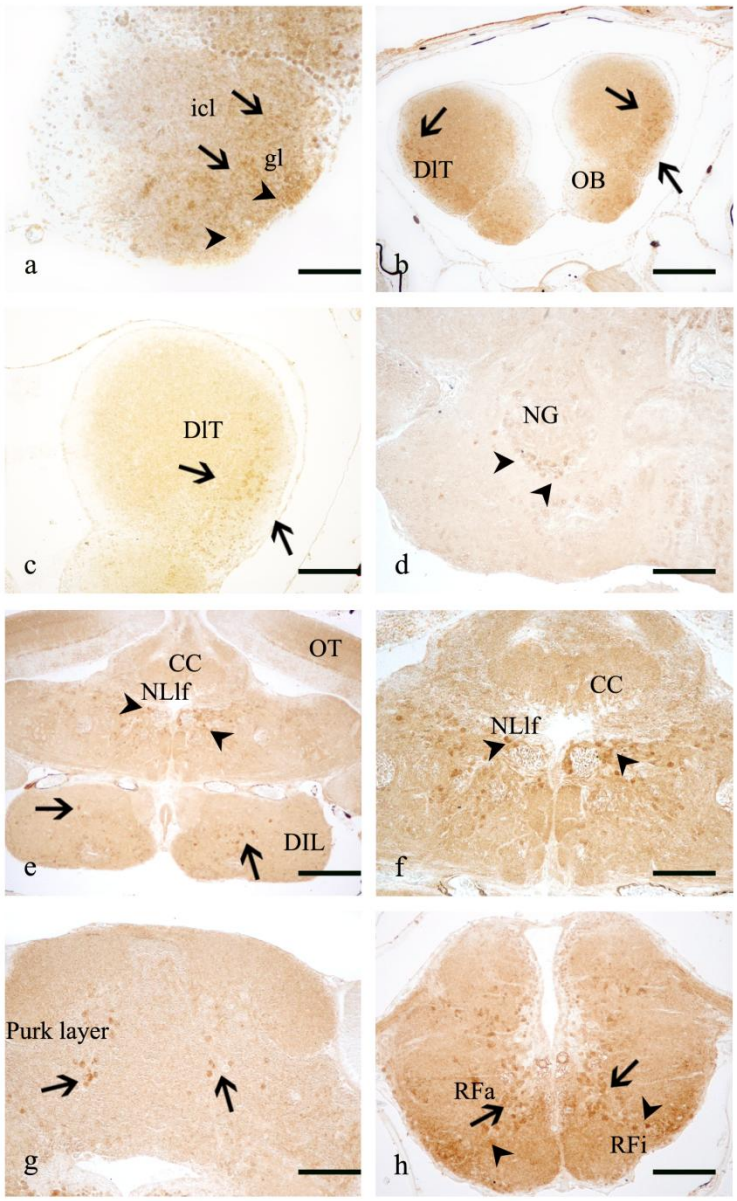


Fig 15. Distribution of NT-4 antigen in the CNS. Transverse forebrain (a-c), midbrain (d-f) and hindbrain (e-h) sections. a. NT-4 protein distributed in few cells of the inner cell layer (icl) (arrows) and in many cells of the glomerular layer (gl) (arrowheads) of the olphactory bulbs (OB). **b.** NT-4 protein distributed in neurons and fibers of the dorso-lateral telencephalon (DlT) (arrows). **c.** High magnification of NT-4 protein distributed in neurons and fibers of the dorso-lateral telencephalon (DlT) (arrows). **d.** NT-4 protein distributed in neurons along the border (arrowheads) of glomerular nucleus (NG). **e.** NT-4 protein distributed in neurons of the diffuse nucleus of inferior lobe (arrows) of hypothalamus (DIL) and in neurons of the nucleus of the medial longitudinal fascicle (NFlm) (arrowheads). **f.** NT-4 protein distributed in neurons of the nucleus of the longitudinal fascicle (NFlm) (arrowheads). **g.** NT-4 protein distributed in Purkinje cells (arrows) of the body of cerebellum. **h.** NT-4 protein distributed in neurons of the anterior (RFa) (arrows) and intermedial reticular formation (RFi) (arrowheads). Scale bars: **a-d-f** = 25 µm; **b** = 100 µm; **c-d-f-g-h** = 50 µm.

4.6 NT-6

4.6.1 ISH (Table 11)

Telencephalon

In the olphactory bulbs, numerous labeled cells were seen in the glomerular and external cell layers (Fig 16a).

In the telencephalic lobes, the expression pattern of NT-6 mRNA was characterized by labeling in the dorsal telencephalon: intense in the dorso-dorsal and dorso-central areas (Fig 16b) and moderate labeling in the dorso-lateral and dorso-medial (Fig 16b) areas. Also in the ventro-lateral area of the ventral telencephalon NT-6 expression was detected. In the dorsal area of posterior telencephalon, a group of neurons was intensely stained.

Cells lining the ventricle, mainly of the central and ventral areas, were moderately labeled.

Diencephalon

NT-6 mRNA expression was intensely detected in the nuclei of preoptic area, either parvo- and magnocellular portion.

In the epithalamus, weak labeling was detected in the small packed cells in the ventral part of habenular nuclei. In the thalamus, intense staining was observed in the anterior thalamic nucleus and in the ventral thalamic nucleus (Fig 16c). Moreover, positive glial cells lining the ventricle were positive (Fig 16c).

Caudally and in proximity of the ventricle, in the dorsal hypothalamus moderate labeling in some large neuron was seen. In the diffuse nucleus of inferior lobe of hypothalamus, a peculiar expression pattern was observed: positive cells lining the margin and neurons in the central portion of the lobe expressed intense NT-6 mRNA (Fig 17c). Posterior, in the diencephalon, strong labeling was observed in the neurons of central nucleus (Fig 16d).

In the optic nerve, grouped cells showed intense staining.

Mesencephalon

In the OT, lower signal was detected in the PGZ (Fig 16e) and intense probe signal was seen in the outer layer of the SWGZ (Fig 16e). Few fibers and neurons were dispersed in the other layers. In the longitudinal tori, intensely NT-6 mRNA labeled fibers were seen in the most apical part, at the margin with the OT, and numerous positive cells were detected mainly in the most ventral part (Fig 16f). In the most caudal part of OT, the cells lining the margin between the OT and tegmentum were intensely labeled (Fig 17a).

In the tegmentum, moderate labeling was seen in neurons bordering the margins of the glomerular nucleus, whereas intense staining was observed in the neurons of the horizontal commissure (Fig 17b), which project toward the ventricle. More medially, a positive probe signal was detected in the cytoplasm of some neurons of the nucleus of medial longitudinal fascicle. Densely packed cells, concentrically arranged, were moderately labeled in the semicircular tori.

NT-6 expression was observed in the nucleus of trigeminal nerve. Posteriorly, moderate labeling was seen in the fibers of the tecto-cerebellar tract and in the lateral nucleus of valvula.

Rhombencephalon

In cerebellum, NT-6 mRNA was mainly localized in Purkinje cells of the valvula and of the body (Fig 17c). The labeling was seen also in the upper part of the cerebellum, in cytoplasmatic processes of radial glial cells (Fig 17c). Few positive cellular elements were labeled in the cerebellar crista.

In medulla oblongata, the expression pattern was characterized by staining in the neurons of octavolateral area, and in neurons of the anterior (Fig 17c,d) and intermedial (Fig 17c) reticular formation .

Table 11*

NT-6 *ISH*		
TELENCEPHALON		
olphactory bulbs	glomerular layer	++
	external cell layer	++
dorsal telencephalon	dorso-dorsal	+++
	dorso-lateral	+
	dorso-medial	+
	dorso-central	+++
	dorso-posterior	+
ventral telencephalon	ventro-ventral	+
ventricle		+
DIENCEPHALON		
preoptic area	preoptic nucleus	++
epithalamus	habenular nuclei	+/-
thalamus	anterior thalamic nucleus	++
	ventral thalamic nucleus	++
hypothalamus	dorsal hypothalamus	+
	diffuse inferior lobe	++
pretectum area	cortical nucleus	++
optic nerve		+
ventricle		++
MESENCEPHALON		
optic tectum	PGZ	++
	SWGZ	+
longitudinal tori		+
tegmentum	glomerular nucleus	+
	horizontal commissure	++
	nucleus of medial longitudinal fascicle	+/-
	semicircular tori	+
	nucleus of V	++
	tecto-cerebellar tract	+
	lateral nucleus of valvula	+
RHOMBENCEPHALON		
cerebellum	valvula	++
	body	++
	cerebellar crista	+/-
medulla oblongata	nucleus of octavolateral area	+
	reticular formation (anterior, medial)	++
* **+++ strong; ++ mild; + weak; +/- very weak**		

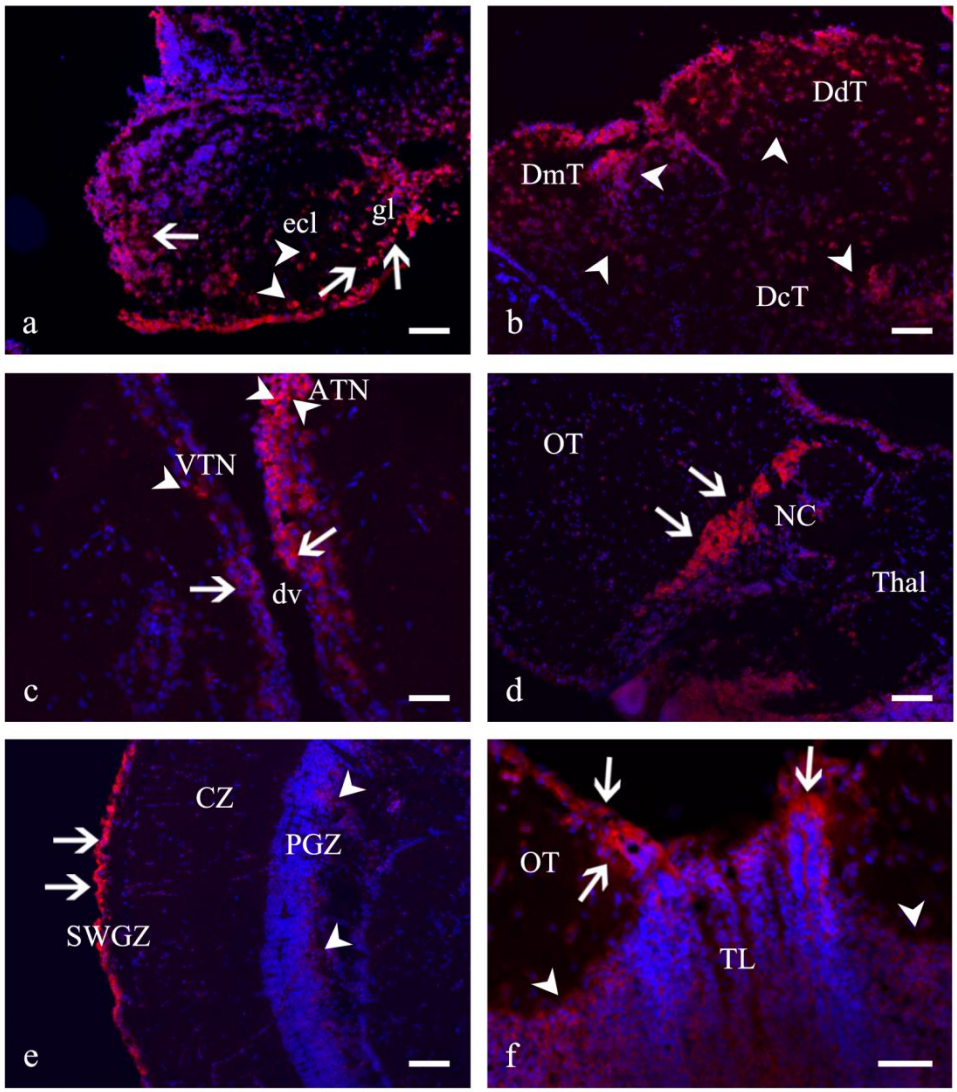


Fig 16. Expression of NT-6 mRNA in the CNS. Transverse forebrain (a-d), midbrain (a-f) sections. a. NT-6 mRNA expressed in cells of the glomerular (gl) (arrows) and external cell layers (ecl) (arrowheads) of olphactory bulbs. **b.** NT-6 mRNA expressed in neurons of the dorso-dorsal (DdT), dorso-medial (DmT) and dorso-central (DcT) areas of telencephalon (arrowheads). **c.** NT-6 mRNA expressed in neurons of the anterior (ATN) and the ventral thalamic nucleus (VTN) (arrowheads), and in glial cells (arrows) lining the diencephalic ventricle (dienc ventr). **d.** NT-6 mRNA expressed in neurons of cortical nucleus (arrows). **e.** NT-6 mRNA expressed in fibers of the outer layer of the superficial white and grey zone (SWGZ) (arrows) and in cells of the periventricular grey zone (PGZ) (arrowheads) of the OT. **f.** NT-6 mRNA expressed in fibers of the the most apical part of longitudinal tori (TL) (arrows), at the margin with the optic tectum (OT) (arrows), and in cells of the most ventral part of TL (arrowheads). Scale bars: **a-b-e** = 200 µm; **c-d-f** = 100 µm.

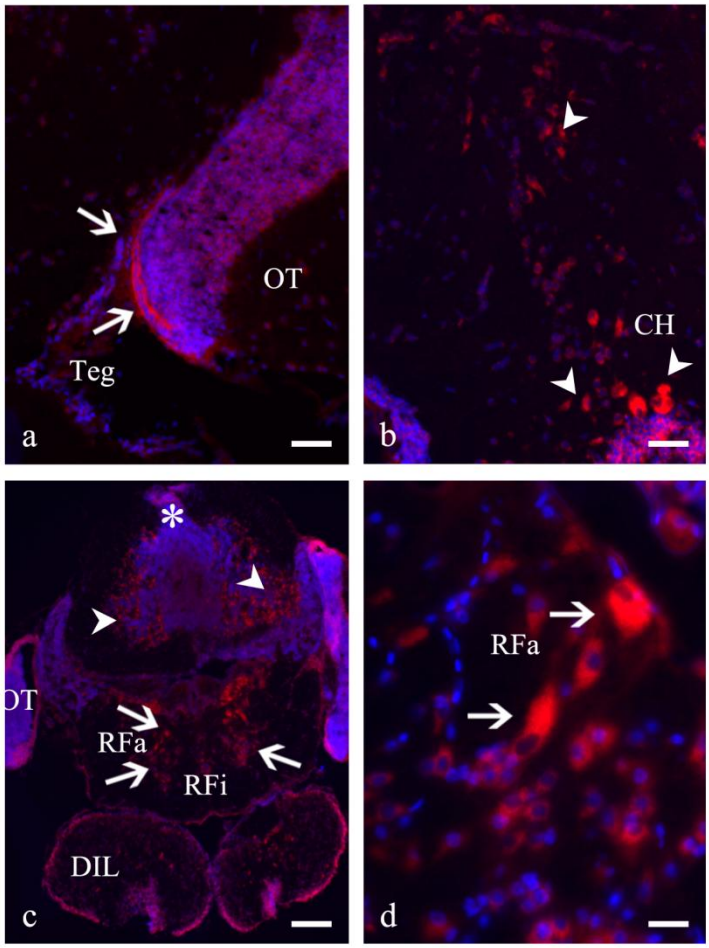


Fig 17. Expression of NT-6 mRNA in the CNS. Transverse midbrain (a-b), hindbrain (c-d) sections. a. NT-6 mRNA expressed in the cells lining the margin between the optic tectum (OT) and tegmentum (Teg) (arrows). **b.** NT-6 mRNA expressed in the neurons of the horizontal commissure (CH) (arrowheads). **c.** NT-6 mRNA expressed in Purkinje cells (arrowheads) and in radial glial cells (arrowheads) at the uppermost part of cerebellum, in neurons of the anterior (RFa) and intermedial (RFi) reticular formation (arrowheads), in cells along the margin and in the central part of the diffuse nucleus of inferior lobe (DIL). **d.** High magnification of neurons of anterior reticular formation (RFa) (arrows) expressing NT-6 mRNA. Scale bars: **a-b** = 100 µm; **c** = 400 µm; **d** = 50 µm.

4.7 Phylogenetic analysis

The built phylogenetic tree is shown in Fig 18.

The tree obtained yielded a congruent phylogeny for both nucleotide and aminoacid sequences of BDNF. This gene tree fits the “species” tree expected on the basis of the accepted species relationships, indicating that the genes sampled are indeed orthologous, and therefore suited for subsequent analyses on substitution rates.

With the regards to the construction of the tree of all other neurotrophins, the tree substantially agree with those previously reported: it highlights that the four vertebrate neurotrophins were generated by two subsequent duplication events of an ancestral gene, one involving the generation of the NGF/NT-3 and another involving generation of BDNF/NT-4/5 groups after the split of lamprey from the common vertebrate lineage (Hallböök *et al.*, 1999, 2001; Lanave *et al.*, 2007).

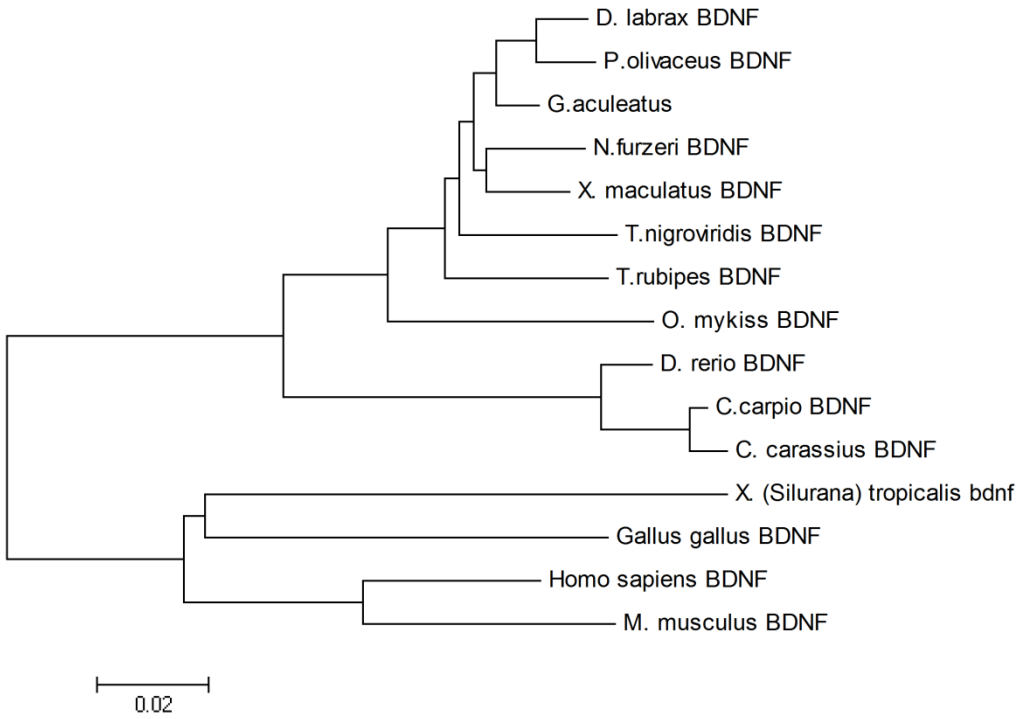


Fig 18. The phylogenetic tree of nucleotide and aminoacid BDNF sequences was reconstructed by MEGA5. For *N. furzeri*, the nucleotide sequence is listed in 3.4.5.

5. DISCUSSION

The current study describes an atlas of the brain of *N. furzeri* in the transverse plane. The brain atlas is also useful for comparative studies, and is propaedeutic for neuroanatomic investigations.

This survey reports, for the first time, the presence and distribution of neurotrophins in the brain of *N. furzeri*, a teleost which is a new aging model. In addition, it is also the first microscopical localization of NT-6 in teleosts and the first description of NGF and NT-4 in teleost brain. Except for NT-3, which is practically lacking, all neurotrophins were widely diffused throughout the different regions of the brain, preferentially localized in neurons and other cellular populations, such as glial cells lining the ventricles or immature cells (neuroblasts/glioblasts) of supposed neurogenic areas.

5.1 Methodological approaches: *in situ* hybridization technique and immunohistochemistry

Two methodological approches were used to investigate neurotrophin distribution: ISH and IHC techniques.

ISH is a commonly used sensitive technique to identify, localize, and study regulation of mRNA expression in brain tissue sections.

To detected BDNF mRNA distribution, two different types of probes for the ISH experiments were employed: locked nucleic acid (LNA) and DNA oligonucleotides probes. The LNA is an engineered nucleic acid analogue, containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA-mimicking sugar conformation (Petersen and Wengel, 2003; Shantanu and Deepak, 2006). The hybridization properties of LNA oligonucleotides have been evaluated in several different sequences, ranging from six to 20-nucleotide long oligomers with various levels of LNA content (Wahlestedt *et al.*, 2000). Oligonucleotides containing LNA exhibit thermal stabilities toward complementary DNA (Wengel, 1998) and RNA and toward

complementary DNA and RNA and exhibit excellent mismatch discrimination. Moreover, LNA probes enhance hybridization efficiency, (Kubota *et al.*, 2006), hybridization specificity and duplex stability (Mctigue *et al.*, 2004). LNA probes can also alternatively detect spliced exons (Darnell *et al.*, 2010). Despite these attributes, the use of LNAs to detect mRNAs is up to date very limited. Darnell *et al.* (2010) investigated the use of LNA containing DNA probes for whole mount ISH detection of mRNAs. However, the use of LNA probes to study mRNAs expression on tissue sections has not been reported. To evaluate the feasibility of using short LNA containing oligonucleotides as hybridization probes for detection of mRNAs by sections, ISH LNA probes were designed and were tested in parallel with the DNA oligonucleotide probes. Hence, a comparison between the two probes was carried out to confirm the expression of the BDNF mRNA and to further validate the employment of the LNA probes for detecting mRNAs coding for structural, signaling, and transcriptional regulatory proteins. Both the probes revealed an analogous expression pattern throughout the CNS, by labeling the same encephalic nuclei. The ability of short LNA probes to detect mRNA sequences allows for the localization of specific splice variants and allows probes to be designed in silico and chemically synthesized, eliminating the need for a cDNA template.

Thus, for the detection of NGF, NT-3, NT-4 and NT-6 mRNAs, only LNA probes were employed.

Regarding IHC, for this study commercially available antibodies against NGF, BDNF, NT-3 and NT-4 were employed. In pairwise alignments, the amino acid sequence for *N. furzeri* BDNF displayed about 86–97% identity with BDNF sequences from other teleost fish, *Xenopus*, chicken, rat, pig and human. Concerning NGF, NT-3, NT-4, the overall identity of aminoacid sequences in pairwise alignments show reduced percentage. Particularly, NGF, NT-3 and NT-4 share an aminoacid sequence identity of about 60% between mammals and teleosts. However, the antisera employed were directed against well conserved sequences.

5.2 BDNF

5.2.1 Comparative analysis of BDNF localization in N. furzeri brain and other vertebrate species

Studies on neurotrophins in teleosts are focused mainly on the occurrence of BDNF and its receptor TrkB over different tissues, ranging from developmental to adult stages (Hashimoto and Heinrich, 1997; de Girolamo *et al.*, 1999, 2000; Lucini *et al*., 1999, 2001, 2002; Hannestad *et al*., 2000; Catania *et al*., 2007; Germanà *et al.*, 2004, 2010a, 2010b;). In teleost CNS, data on the presence of BDNF were reported in the neuroanatomical description of the expression of mRNA BDNF throughout the brain of adult *Anguilla anguilla* (Dalton *et al.*, 2009), and the expression of BDNF and its receptor TrkB after spinal cord injury in *Anguilla anguilla* (Dalton *et al.*, 2009). Additionally, BDNF immunoreactivity-like was reported in the brain and retina of *Cichlasoma dimerus* (Vissio *et al.*, 2008).

Coherently with the results reported in the teleost species (Dalton *et al.*, 2009; Vissio *et al.*, 2008), the occurrence of mRNA BDNF expression and BDNF protein was detected in all region of the brain of *N. furzeri.* Comparing the occurrence of BDNF in *N. furzeri* brain to the pattern of expression of BDNF in *A. anguilla* brain, a cospicuous number of corresponding nuclei and zones showed BDNF expression in both *N. furzeri* and *A. anguilla*.

In the telencephalon of *A. anguilla*, both in olphactory bulbs and telencephalic hemispheres, rostral and caudal parts, the BDNF mRNA expression was widely described in all regions of telencephalon and olphactory bulbs. Few scattered cells were lightly labeled in the entopeduncolar nucleus of *A. anguilla* (Dalton *et al.*, 2009). Interestingly, the neurons of the entopeduncolar nucleus of *N. furzeri* brain were positive to BDNF protein, whereas the BDNF mRNA was not detected. Some dissimilarities of the BDNF mRNAs expression, between *N. furzeri* and *A. anguilla*, were observed in the diencephalon. In *A. anguilla*, BDNF mRNA was detected in the periventricular zone of hypothalamus, caudal and ventral, in the subcommissural and posteriorly paraventricolar organ, and in preglomerular complex. In *N. furzeri*, there was nor BDNF mRNA or protein, detectable in those brain structures. The overall pattern of BDNF mRNA

expression in mesencephalon and rhomboencephalon did not reveal significant differences between *A. anguilla* and *N. furzeri*. The most relevant difference is attributable to the detectability of BDNF in the lateral nucleus of cerebellar valvula and Mauthner-like cell of *A. anguilla*, while no positive BDNF products were found in the analogous structures of *N. furzeri* CNS. The different sites of distribution could be related to different experimental procedures, but also to species specific characteristics, bearing in mind that *N. furzeri* and *A. anguilla* are two species philogenetically distant to each other.

The expression pattern detected in *N. furzeri* brain seems to be consistent also with the data available on the expression of mRNA BDNF in mammals (Conner *et al.*, 1997), avian (Herzog and von Batheld, 1998) and amphibian brain (Duprey-Diaz *et al.*, 2002), taking into account the neuroanatomical differences and homologies relative to the species. Particularly, homologies between mammals and teleosts brain have been widely discussed. Several hypothesis have been formulated on the equivalent of teleostean telencephalon. However, the common starting point was the well accepted idea that the teleostean telencephalon, during the development, undergoes a eversion process instead of an evagination, as occurs in mammals. Consequently, the dorsal telencephalic areas correspond to the mammalian pallial formation and the ventral one to the subpallium. Specifically, Wullimann and Mueller (2004) found that the teleostean dorso-lateral telencephalon would be the mammalian homologous of medial pallium or hippocampus; the everted part of dorso-medial telencephalon would correspond to the dorsal pallium of other vertebrates (isocortex homologue); the pallial masses ventral to dorso-lateral would not be everted. Furthermore, the adult posterior zone of dorsal telencephalic area would be the lateral pallium of other vertebrates (i.e. the olfactory cortex homologue); the midline portion of the medial zone of dorsal telencephalic area would correspond to the homologue of the pallial amygdala. The adult dorsal and ventral areas of the ventral telencephalon would correspond respectively to the striatum and septum homologues. This hypothesis support the theory of the partial eversion of teleostean telencephalon, contrasting the complete eversion theory (Nieuwenhuys, 2009). According to the latter theory, the dorso-lateral telencephalon is homologous to the medial pallial formation, the ventro-dorsal

and ventro-central to the striatal formation, and the ventro-ventral and ventro-lateral to the septal formation.

Bearing in mind the homologies, in rodents CNS, BDNF mRNA expression was detected in the amygdala, hippocampus and some other basal nuclei where BDNF seems involved in the functional role of the structure (Conner *et al*., 1997). Thus, it is presumable that BDNF in *N. furzeri* brain might act and regulate functional activity of the telencephalic neurons. Furthermore, in rodents occurrence of BDNF mRNA was seen in several dopaminergic thalamic nuclei (Conner *et al*, 1997). In *N. furzeri*, putative dopaminergic nuclei, equivalent to the mammalian ones, such as the anterior and ventro-thalamic nuclei, and the preoptic nuclei, resulted positive to BDNF. These nuclei, in mammals as well as in teleosts, represent an important sensory relay station, although the dopaminergic ascending system seems to have different organization: in fact, in teleost the ascending system origins from the rostral encephalic region, precisely from diencephalon (Rink and Wullimann, 2001; Rink and Wullimann, 2002), whereas in mammals from mesencephalic tegmentum.

In *N. furzeri*, mRNA BDNF expression was observed in the dorsal and ventral habenular nuclei respectively considered as homologues of the mammalian medial and lateral habenulae (Amo *et al.*, 2010) where Conner *et al*. (1997) detected BDNF transcript.

Moreover, in *N. furzeri*, intense labeling of BDNF was seen in the semicircular tori, an important station of sensory inputs. Based on comparative studies, these symmetric structures correspond to the mammalian inferior colliculus, which also showed BDNF expression (Conner *et al.*, 1997).

More caudally, in the cerebellum of *N. furzeri* BDNF intense labeling was observed in Purkinje neurons of the valvula, body and caudal lobe, and in cells of the granular layer. In rat (Conner *et al.*, 1997) as well as in human (Quartu *et al.*, 2010), the BDNF expression was not reported in adult cerebellum, whereas it was described in the cerebellum of both human (Quartu *et al.*, 2010) and rat (Rocamora *et al.*, 1993) during postnatal development.

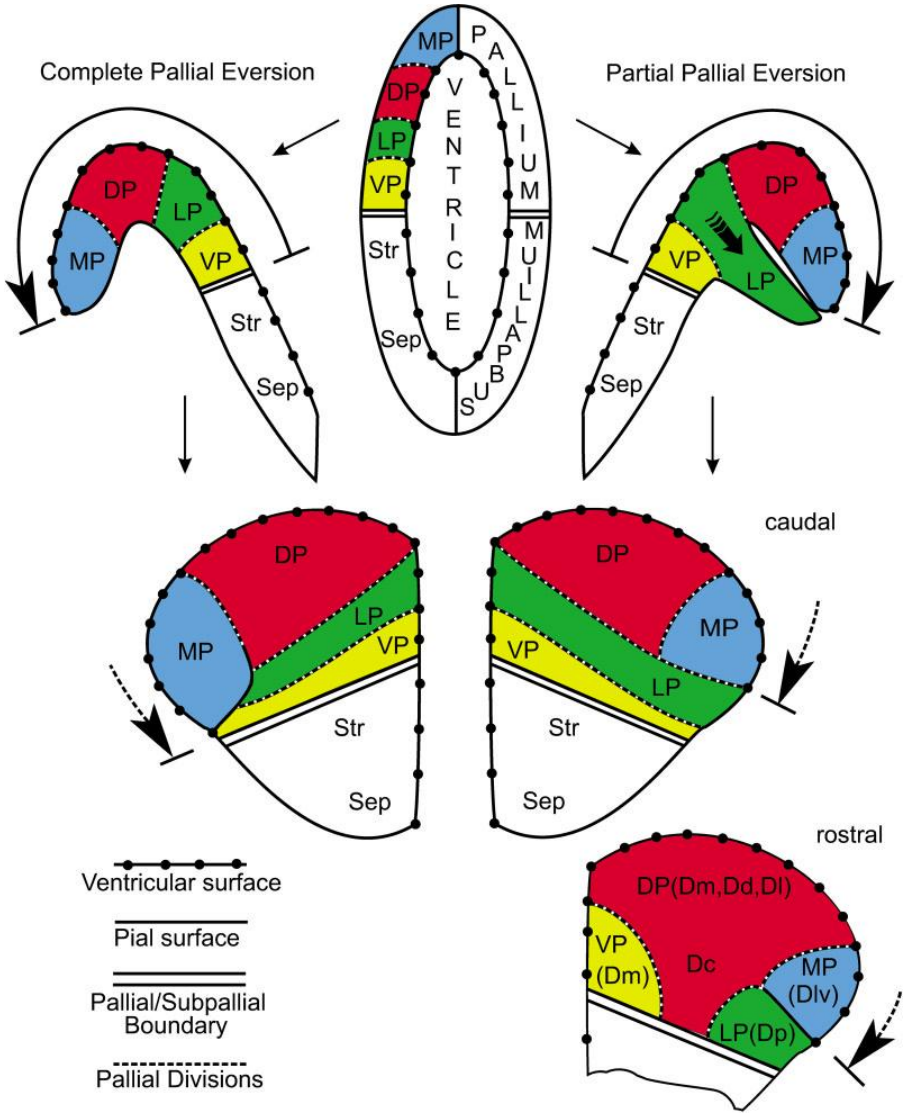


Fig 19. Complete (left) vs. incomplete (partial; right) pallial eversion model in teleosts and hypothetical relationship of four tetrapod (medial, dorsal, lateral, ventral) pallial zones to adult teleostean pallial zones. **Abbreviations:** MP medial pallium; DP dorsal posterior thalamic nucleus; LP lateral pallium; VP ventral pallium; Str striatum; Sep spetum; Dm medial zone of area dorsalis telencephali; Dd dorsal zone of area dorsalis telencephali; Dl lateral zone of area dorsalis telencephali; Dc central zone of area dorsalis telencephali; Dlv latero-ventral area dorsalis telencephali; Dp posterior zone of area dorsalis telencephali (Wullimann and Mueller, 2004).

Strong BDNF labeling was also appreciated in the nuclei of reticular formation in the CNS of *N. furzeri*. The reticular formation is a descending supraspinal system, which modulate a range of motor behaviors that include swimming, startle responses, posture adaptation and walking in land vertebrates. Interestingly, in mammals, the teleost equivalent of reticular formation did not reveal BDNF occurrence.

In *N. furzeri* as well as in the eel, intense BDNF labeling was detected in brain structures involved in the visual system, such as nuclei of preoptic area, preglomerular and glomerular nuclei, optic tectum, semicircular tori. BDNF, as well as other neurotrophins, influence proliferation, neurite outgrowth and survival of cells in the visual system in vitro and in vivo (for a review see von Bartheld, 1998). Therefore, it is likely to hypothesize a functional role of BDNF in *N. furzeri* visual system as well as an involvement in terms of regeneration and proliferation activities.

5.2.2 mRNA and protein distribution: evidence of anterograde and retrograde transport?

BDNF mRNA and protein were often localized in the neuronal soma, and BDNF protein was particularly visualized in numerous fibers forming diffused mesh or running together in fascicula. The high number of fibers positive to BDNF protein represents clear clues for an active transport of BDNF along neuron terminals that, by the obtained results, could be a retrograde as well as an anterograde transport. Retrograde transport along the axon represent the traditional view of neurotrophic factor biology, but it is now largely accepted that trophic factors, and particularly BDNF, can move anterogradely along axons and be transferred to the postsynaptic target cells (for a review see Caleo and Cenni, 2004). Some fibers were also positive to BDNF mRNA, although less numerous than BDNF protein, suggesting in adult *N. furzeri* brain a translational activity which is not limited to the soma, but also occurs at dendritic levels as previously described in mammals. BDNF, as other molecules involved in synaptic plasticity, can be delivered in form of silent mRNA at synapses where it is further locally translated (for a review see Tongiorgi, 2008).

Many nuclei showed labeling to both BDNF mRNA and protein, like parvocellular and magnocellular, anterior thalamic, central, dorsal tegmental and glomerular nuclei. Although investigating the neuronal co-localization of ISH and IHC is beyond the purpose of this study, it is quite presumable that neurons belonging to the same nucleus both transcribe and translate BDNF. However, there are nuclei whose neurons expressed only BDNF mRNA or only the protein. Probably the localization of mRNA is sufficient to indicate that translation occurs although the protein was not detected, likely for its paucity. Indeed, BDNF protein seemed to be stored in *N. furzeri* brain at very low levels, because only a very sensible method such as EnVision was able to detect BDNF, while other methods, as PAP, ABC, failed to reveal immunoreactivities (unpublished data). The finding showing only BDNF protein in the nuclei could reflect true lack of mRNA transcription, and consequently the neurons should uptake BDNF in intersynaptic space by means of retrograde transport along the axon toward the soma. Furthermore, among these nuclei, ventromedial thalamic nucleus, paraventricular organ and solitary fascicle nucleus are very close to ventricles and could act as cerebrospinal fluid (CSF) contacting neurons. These latter send dendritic processes into the internal CSF and take up substances from the brain ventricles and, then, transmit them through perikarya to synaptic zones of various brain areas. Besides, in fish CSF contacting neurons were reported to be more numerous than in mammals (Vigh *et al*., 2004). Because in mammals CSF contains BDNF and its level is related to neurological diseases (Papaliagkas *et al*., 2010; Kizawa-Ueda *et al*., 2011), it could be intriguing to retain that neurons of these nuclei take up BDNF from CSF and then release it by axonal anterograde transport.

5.2.3 BDNF: possible implications in neurogenesis and neuroregeneration processes

The cellular proliferation is particularly pronounced in the dorsal telencephalon, preoptic area of the diencephalon, longitudinal tori, optic tectum and in all cerebellar subdivisions of some fish, such as brown ghost (Zupanc and Horschke, 1995), gilt head sea bream (Zikopoulos *et al.*, 2000), goldfish (Meyer, 1978; Raymond and Easter, 1983), guppy (Kranz and Richter, 1970), stickleback

(Ekström and Ohlin, 1995), and zebrafish (Zupanc *et al*., 2005). Preliminary studies in *N. furzeri* CNS also seem to suggest a similar pattern of distribution of mitotic cells (unpublished data). The obtained results on distribution of BDNF in *N. furzeri* appeared often present in areas of neurogenesis. In particular, all nuclei of the preoptic area are reported to show mitogenic cells, and both magnocellular and parvocellular preoptic nuclei displayed BDNF. In the optic tectum, mitogenic areas are identified mainly in the periventricular grey zone and superficial layers, where BDNF mRNA and protein were particularly dense.

In the cerebellum body, BDNF was present in the molecular layer and limited to a medial zone of the granular layer. Zupanc *et al.* (2005) described proliferation zones in the cerebellum body, restricted to areas at and near the midline in the dorsal and ventral molecular layers. The new cells migrate from the sites of origin to specific target areas (Zupanc *et al.*, 1996) which are the granule cell layers in both the cerebellum body and the valvula, lateral and medial parts.

Taking in account that BDNF has been identified as a molecule that contribute to stimulate rodent adult neurogenesis of olfactory bulbs (subependymal zone of the lateral wall) and hippocampus (subgranular zone), regulating the proliferation, migration and survival of newly born neurons (for a review see Bath and Lee 2010; Bath *et al.*, 2011), the present observation regarding the brain of *N. furzeri* strongly suggest an involvement of BDNF in neurogenic process. These results could open new perspectives for better understanding the biological function of adult neurogenesis in lower vertebrates models, like *N. furzeri*, for aging research in view of studying treatment of neurodegenerative diseases.

5.3 NGF

5.3.1 Comparative analysis of NGF localization in N. furzeri and rodents brain

The widespread expression of NGF in the telencephalon of *N. furzeri* recalls the localization of NGF in the corresponding neuroanatomical structures of mammalian forebrain. NGF is synthesized and secreted by cells in the cortex and hippocampus and acts on neurons of basal forebrain nuclei by binding TrkA and

p75 (Shelton and Reichardt, 1986; Conner and Varon, 1992; Conner *et al*., 1992; Talamini and Aloe, 1993; Niewiadomska *et al.*, 2011). The neurons of mammalian basal forebrain nuclei are recognized as cholinergic. A recent paper on the cholinergic teleostean system confirms that the neurons of the ventral telencephalic areas (corresponding to the mammalian subpallium) are cholinergic (Mueller *et al.*, 2004). The presence of NGF in these areas of *N. furzeri* encephalon suggests that also in teleost NGF may act as a target-derived trophic factor for cholinergic neurons (Talamini and Aloe, 1993). The cholinergic neurons of the basal forebrain nuclei have been assumed undergo moderate degenerative changes during aging, resulting in cholinergic hypofunction that has been related to the progressing memory deficits with aging. In pathological aging, such as Alzheimer's disease, the above-mentioned degenerative changes are more relevant. The significant cholinergic cell loss during aging has been challenged. In particular, the cholinergic dysfunction may be related to imbalances in the expression of NGF as well as to changes in acetylcholine release, high-affinity choline uptake, and alterations in muscarinic and nicotinic acetylcholine receptor expression (for a review see Schliebs and Arendt, 2011). Hence, the occurrence of NGF molecule in the cholinergic neurons in the adult CNS of *N. furzeri* might represent a baseline study for understanding the molecular mechanism of neurodegeneration diseases, such as Alzheimer disease.

5.3.2 NGF: spatial expression and distribution of mRNA and protein in the CNS

ISH and IHC were employed to map the cellular localization of NGF mRNA and protein in the adult brain of *N. furzeri*. Not in all encephalic regions, the distribution of NGF mRNA overlapped with the distribution of NGF protein.

NGF mRNA expression was rather intense in the telencephalic region, specifically in the dorsal and ventral areas of telencephalon. NGF protein showed a similar pattern of distribution but appeared less distributed in the ventral areas. In the diencephalon, neurons of the nuclei of the preoptic area, the habenular nuclei, the ventral thalamic nucleus and the diffuse nucleus of inferior lobe of hypothalamus were positive to both NGF mRNA and protein. Moreover,

the mRNA was expressed in the entopeduncolar nucleus, whereas the protein was present in the pretectal nucleus. Deep differences were noted in the mesencephalon, where the mRNA was widely appreciated, as it resulted expressed in all major structures of mesencephalic region such as optic tectum, longitudinal tori, central nucleus, glomerular nucleus, semicircular tori, longitudinal and medial fascicles in contrast to the protein, whose localization was limited to glomerular nucleus, semicircular tori, medial longitudinal fascicle. More caudally, in the rhombencephalon, the localization of both NGF mRNA and protein was seen in Purkinje cells of the cerebellum body, in the medial and inferior nuclei of reticular formation and in the nucleus of VIII nerve. The mRNA expression was detectable in the fibers of gustatory tract. However, in certain nuclei, NGF mRNA and protein overlapped. For example, in the glomerular nucleus NGF mRNA and protein were observed in neurons localized along the inferior external margin of nucleus, or in the cerebellum, where Purkinje cells positive to NGF mRNA and were also immunopositive to the protein. It is likely to hypothesize that those neurons act as targets-cells as well as storage for NGF.

In rat brain, the detection of NGF antigen by immunohistochemical methods was seen in restricted neuronal populations among all synthesizing NGF (Conner and Varon, 1992; Conner *et al.*, 1992). The occurrence of the protein could be due to the retrograde transport and accumulation of NGF from distant sites of synthesis. Moreover, the antigen was identified in locations other than within cell bodies (i.e., the hippocampal mossy fiber region), indicating that the site where a protein is stored need not be within the cell body, where the synthesis occurs (Conner *et al.*, 1992). Therefore, accumulation of a protein at extracellular sites may result from either an anterograde transport down axonal or dendritic arbors of the producing cell, or by the selective binding or uptake and accumulation of protein released into the extracellular space.

A previous study on the distribution of TrKA receptor in the CNS of *N. furzeri* (D'Angelo *et al.*, 2011) described TrkA IR in fibers and rare neurons distributed in the areas of dorsal and ventral telencephalon. Thus, the colocalization of mRNA, protein and receptor could indicate that NGF is synthesized by certain telencephalic neuronal populations and, therefore, NGF might act in an autocrine

manner. Given the high TrkA immunopositivity in fibers throughout the telencephalon and the pattern of NGF molecule expression, it is likely to confirm the active retrograde transport of signaling endosomes carrying NGF bound to activated TrkA (for a review see Campenot, 2009). The retrograde transport could influence the microenvironment by exerting paracrine actions on different neuronal populations, leading to tissue protection, repair, and regeneration.

5.4 NT-3

NT-3: limited protein distribution and no detectable mRNA

The neuroanatomical distribution of NT-3, either the mRNA and protein, was almost absent throughout the whole CNS of *N. furzeri.* Particularly, the mRNA expression was not detectable in any regions. This may be discussed in relation to the technique employed. The LNA probe could have not hybridized properly, although the protocol followed the same steps of all other neurotrophins and, moreover, other experiments carried out on non neural tissues expressed the mRNA. In mammals, the localization of NT-3 is manifested at all developmental stages studied and into adulthood (for a review see Chalazonitis, 1996). In the adult brain of rats, the expression of the NT-3 gene was predominantly confined to the hippocampal formation and cerebellum (Maisonpierre *et al.*, 1990; Ceccatelli *et al.*, 1991 Friedman *et al.*, 1991; Lauterborn *et al.*, 1994), and with a less extent in other brain regions. By contrast, the protein NT-3 was distributed in glia and neurons of numerous brain structures, with the most abundant immunopositivity in the forebrain cortex, hippocampus and cerebellum (Zhou and Rush, 1994). The presence of the NT-3 protein in the adult brain of *N. furzeri* was limited to some specific areas such as hypothalamic region or along the diencephalic ventricle. Repeated trials showed the distribution of the antigen in other structures, such as pituitary gland or some encephalic ganglia. The lower distribution of NT-3 may be attributable to a limited role in the biology of adult CNS of teleosts. The functional role of NT-3 in the teleosts brain deserves more investigations, considering also the lack of available information. In this sense, it is challenging to study the expression during development, so that a comparison in relation to the age could be helpful for better understanding the functional role of NT-3 in the brain of teleosts.

5.5 NT-4

NT-4: restricted spatial expression and distribution in the CNS

NT-4 was moderately expressed in the brain of *N.furzeri*. Although similarities between NT-4 and BDNF, due to the common ancestor gene and the affinity to binding to the same TrkB receptor, the occurrence of NT-4 molecule in the CNS of *N. furzeri* was rather limited to few regions. The highest rate of NT-4 products was appreciated in the telencephalon, where both mRNA and protein were localized. The occurrence was detectable in some nuclei involved in the visual pathways, such as the preoptic nucleus and the pretectal area, as well as in the Purkinje cells of the cerebellum, in the reticular formation and in some other nuclei of medulla oblongata. However, it is noteworthy that there was no equal correspondence between mRNA and protein in the same neuronal populations. Indeed, a number of nervous structure which did not express detectable NT-4 mRNA showed IR to NT-4 antibody, which recognizes internal region of NT-4 of fish origin. Differences in the localization of mRNA and protein were also observable in the results of the other neurotrophins, but in regard to NT-4 these differences appeared more distinct and evident. Alike in mammals (Friedman *et al.*, 1998), in *N. furzeri* encephalon the protein distribution was observed in neuronal populations where no mRNA was detectable. For example, the longitudinal tori and the glomerular nucleus showed the presence of antigen but not the mRNA. It is presumable that the site of action might not be the site of snthesis of the factor, or that the protein was transported to the site of action. This observation could suggest that also in teleosts NT-4 might be involved in axonal retrograde and anterograde transport, and even in transcellular signalling (Wirth *et al.*, 2005). In this way NT-4 supports afferent and efferent neurons in different target areas.

In mammals, studies demonstrated that the amount of NT-4 in the brain, irrespective of the brain regions and post-natal ages, were far lower than those in other organs (Timmusk *et al.*, 1993; Katoh-Semba *et al.*, 2003). Even though the levels were lower in rat brain, NT-4 seems to have specific functions in the individual brain regions, because significant changes in the NT-4 concentration with post-natal development of the brain were observed in several regions.

Interestingly, in rat CNS, NT-4 showed changes in the concentration related to age. While after birth the level of NT-4 in thalamus considerably decreased (Katoh-Semba *et al.*, 2003), marked increases in levels of NT-4 with post-natal age were observed in layer 5 and 6 of the neocortex, in the olfactory bulb, the hypothalamus, and the brain stem (Katoh-Semba *et al.*, 2003). Therefore, in mammals NT-4 plays a role in the CNS either in development and in adulthood. The occurrence of NT-4 level in the adult brain of *N. furzeri* indicates that the biological function of the neurotrophin in specific nervous structures and neuronal populations remains to be elucidated.

5.6 NT-6

NT-6: evidences of mRNA expression

NT-6 expression in the brain of *N.furzeri* represents the first neuroanatomical description in teleosts. NT-6 was first identified in the teleost fish *Xiphophorus* as a new member of the neurotrophin gene family. A supposed, evolutionarily close neurotrophin, NT-7, was isolated and identificated in other teleost species, zebrafish (Nilsson *et al.*, 1998) and carp (Lai *et al.*, 1998). However, molecular studies have demonstrated that in fish only NT-6 exists (Dethleffsen *et al.*, 2003), and that the mature proteins of NT-6 and NT-7 genes are closely related to NGF, since they have evolved from a common ancestor after a single "fish specific" duplication of NGF (Dethleffsen *et al.*, 2003).

Up to date, the data on NT-6 are very limited. In this survey, only mRNA detectability was reported, since there are not commercially available antibodies. In *Xiphophorus*, NT-6 transcripts are described to be expressed during the embryonic development as well and in the adulthood. It was found in the brain, gill, liver and eye and with weak expression in heart, liver, skin, spleen and skeletal muscles (Götz *et al.*, 1994). In the brain of *N. furzeri*, NT-6 resulted largely localized throughout different brain regions. The survey reported an almost ubiquitous distribution of cells expressing NT-6 mRNA within the CNS. Interestingly, a peculiar pattern of NT-6 mRNA expression was observed in certain regions. For example, in the optic tectum, where the labeling was specifically detected in the cells and fibers along the most superficial margin of

the superficial gray zone. This particular pattern might shed light on the involvement of NT-6 in the visual system of *N. furzeri*, as it has already been hypothesized for other neurotrophins. In mammals, indeed, all neurotrophins play different roles in regulating visual cortical development and plasticity by acting on different neural targets (for a review see Berardi and Maffei, 1999).
Furthermore, in the brain of *Xiphophorus,* Götz *et al.* (1994) detected transcripts in the embryonic valvula of cerebellum, although the cellular population was not identified. In the adult *N. furzeri* cerebellum, transcripts of NT-6 were surveyed in the Purkinje cells of either valvula and body, and in the processes of radial glial cells in the upper portion of cerebellum body. This observation is of great interest, since radial glia represents one of the subpopulation of progenitors in the adult teleostean brain (Pellegrini *et al.*, 2007; Ganz *et al.*, 2010; Rothenaigner *et al.*, 2011; Chapouton *et al.*, 2011) and moreover, relative large numbers of new cells are generated in the cerebellum of fish. It is likely to assume that also NT-6, among several dozens of molecular factors, potentially contribute to the process of neurogenesis and neuroregeneration in teleosts. However, very few is known about the biological and challenging role of NT-6 in fish. Up to date, *in vitro* studies and phylogenetic analysis have been performed aiming at clarifying the biological features of this neurotrophin. *In vitro* studies reported that NT-6 promoted neuronal survival and neurite outgrowth (Li *et al.*, 1997). Phylogenetic analysis of the neurotrophins, performed on mature aminoacid sequences (Hallböök *et al.*, 1991, 1999) supports the notion that NT-6 evolved early in vertebrate history during two rounds of duplication events of an ancestral gene, the gene encoding for NGF/NT-3/NT-6. Further studies have shown that the genomic organization and transcript structure of NGF and NT-6 in the teleost zebrafish share a high similarity with the mouse NGF (Dethleffsen *et al.*, 2003), and suggest that teleost NT-6 has evolved from a common ancestor after a single “fish specific” duplication of NGF. Moreover, like NGF, NT-6 interacts only with the receptor TrkA, although weakly (Götz *et al.*, 1994; Lai *et al.*, 1998). It is of great interest to deepen the role of NT-6, also in relation to NGF.

6. CONCLUSIONS

Model organisms have played a huge part in the history of studies of human genetic disease, both in identifying disease genes and characterizing their normal and abnormal functions. Over the model organisms, fish have lots of advantages. They have similar body plans and similar tissues and organs to humans, and they are much easier and cheaper to look after than the classical rodents models. Furthermore, the development and improvement of the genomic tools have contributed to stimulate interest in fish, and reinforce the usefulness as model organism in the research. In the neurobiology research, fish reflect the main idea that the understanding of the simplest system provides a road map for understanding the fundamental working units in the CNS. This idea was the substrate on which this research has been structured. This study has, at first, been focused on the neuroanatomy of the model employed. Thus, a comprehensive whole brain atlas of *N. furzeri* was constructed as baseline for following experimental procedures. The map of encephalon will potentiate the representation of structural and functional data of future experiments.

The identification and characterization of cDNA fragment complementary to the mRNA sequences which encode the mature form of neurotrophins allowed to study, for the first time, the gene structure and the expression patterns of each neurotrophin in the CNS of the teleost *N. furzeri*. With respect to the expression pattern, the innovative detection of mRNAs by LNA/DNA probes and the compared expression patterns between the LNA/DNA and riboprobes open new perspectives for the applicability of LNA probes for ISH.

Finally, the achieved results on the occurrence of neurotrophins in the brain of *N. furzeri* have added new information regarding neurotrophin biology in vertebrates, and in teleost fish particularly. The main points of interest emerging from this research are that neurotrophins, except NT-3, are all widely expressed in *N. furzeri* brain, and are localized in numerous nuclei and in active proliferating areas. Moreover, the comparison between the distribution of mRNAs and proteins has suggested hypothesis concerning the identification of

neurotrophin synthesis and storage areas, and assumptions regarding their retrograde/anterograde transport.

In conclusion, neurotrophins appear involved in many biological functions of the adult brain of *N. furzeri*.

The comprehension of the role of these molecules in the brain of *N. furzeri* is a formidable tools to investigate the etiology, the course and the potential treatment of neurodegenerative diseases. Neurotrophins, indeed, are markedly involved in Alzheimer disease, Parkinsons' disease, Hungtinton diseas, ALS, mood disorder, thus it appears extremely advantageous the study of neurotrophins in the *N. furzeri* model system.

7. ABSTRACT

In biomedical and veterinary research, fish represent an extraordinary repertoire of models with which to carry out experiments. In the basic neuroscience, fish offer examples that indicate the usefulness of comparative neurobiological investigation in elucidating both normal and abnormal structure and function of the brain of vertebrates, and moreover they possess an enormous potential for the production of new neurons in the adult brain. In aging research, several fish species exhibit gerontological characteristics and provide research opportunities for disease modeling, drug discovery, and regenerative medicine.

This PhD thesis has developed a new teleost model organism *N. furzeri*, which is the shortest-lived vertebrate which can be cultured in captivity. This teleost shows typical signs of aging and represents an ideal model to assess the role of different physiological and environmental parameters on aging and lifespan determination.

The topic of this dissertation is the study of the organization of brain of *N. furzeri* and the neuroanatomical description of the occurrence of neurotrophins. The contruction of the brain atlas is a clear and fundamental guide to the structural and functional organization of the *N. furzeri* CNS, and provides informative reading for subsequent neurobiological surveys. The atlas has been the substratum for the neuroanatomical description of the occurrence of neurotrophins, both mRNA and protein, within the CNS. Neurotrophins are secreted proteins that link nervous system structure and function in vertebrates. They regulate neuronal survival, and regulate patterns of dendrites and axons, synaptic function, memory, learning, and cognition; abnormalities in neurotrophin function underlies neurological disorders. BDNF, NGF, NT-3, NT-4 and NT-6, all members of neurotrophins family, have been identified on expressed sequence tag (EST)-to-genome and genome-to-genome comparisons of *N. furzeri* and other teleosts. ISH and IHC analysis of cells expressing mRNAs for the neurotrophins revealed a specific expression pattern for each of the mRNAs and proteins. The analysis of sites of neurotrophin synthesis was performed with ISH technique using two LNA/DNA and oligonucleotide probes, encoding mature *N. furzeri* neurotrophin protein. The parallel

immunohistochemical investigation, along with specific affinity-purified antibodies, was carried out to generate a detailed mapping of neurotrophin protein throughout the adult *N. furzeri* CNS. All neurotrophins occur in the brain of *N. furzeri*, except NT-3. The obtained results show a high level of correlation between ISH and IHC in the evaluation of neurotrophins in the brain of *N. furzeri*. The data, indeed, have revealed that several nuclei expressing neurotrophin mRNAs were correlated to the neurotrophin specific proteins throughout the CNS; the sites of mRNA synthesis and protein storage have suggested hypothesis on the mechanisms of retrograde/anterograde transport. Moreover, the sites of synthesis have been often identified as active proliferative areas of fish brain, where new neurons are generate or regenerate. The latter observation might suggest a role of neurotrophins in the neurogenic and regeneration activities.

This work provides new aspects of neurotrophic factors in the CNS of vertebrates, and it opens the opportunity to use *N. furzeri* as alternative model to investigate neurotrophin function and model related diseases.

8. REFERENCES

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T. Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res. 85(3):525-35; 2007

Amo R., Aizawa H., Takahoko M., Kobayashi M., Takahashi R., Aoki T., Okamoto H. Identification of the zebrafish ventral habenula as a homolog of the mammalian lateral habenula. J Neurosci.; 30(4):1566-74; 2010

Anken RH., Rahmann H. Brain Atlas of the Adult Swordtail Fish *Xiphophorus helleri* and of Certain Developmental Stages. Gustav Fischer Verlag. 1994

Arevalo JC., Conde B., Hempstead BL., Chao MV., Martin-Zanca D., Perez P. TrkA immunoglobulin-like ligand binding domains inhibit spontaneous activation of the receptor. Mol Cell Biol. 20(16):5908-16; 2000

Barde YA., Edgar D., Thoenen H. Purification of a new neurotrophic factor from mammalian brain. EMBO J 1:549–553; 1982

Bath KG., Lee FS. Neurotrophic factor control of adult SVZ neurogenesis.

Bath KG., Lee FS. Variant BDNF (Val66Met) impact on brain structure and function. Cogn Affect Behav Neurosci. 6(1):79-85; 2006

Bath KG., Mandairon N., Jing D., Rajagopal R., Kapoor R., Chen ZY., Khan T., Proenca CC., Kraemer R., Cleland TA., Hempstead BL., Chao MV., Lee FS. Variant brain-derived neurotrophic factor (Val66Met) alters adult olfactory bulb neurogenesis and spontaneous olfactory discrimination. J Neurosci. 28(10):2383-93; 2011

Benedetti M., Levi A., Chao MV. Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci U S A. 90(16):7859-63; 1993

Berardi N., Maffei L. From visual experience to visual function: roles of neurotrophins. J Neurobiol.;41(1):119-26; 1999

Berkemeier LR., Ozçelik T., Francke U., Rosenthal A. Human chromosome 19 contains the neurotrophin-5 gene locus and three related genes that may encode novel acidic neurotrophins. Somat Cell Mol Genet. 18(3):233-45; 1992

Berkemeier LR., Winslow JW., Kaplan DR., Nikolics K., Goeddel DV., Rosenthal A. Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron. 7(5):857-66; 1991

Bernstein SL., Russell P., Wong P., Fishelevich R., Smith LE. Heat shock protein 90 in retinal ganglion cells: association with axonally transported proteins. Vis Neurosci. 18(3):429-36; 2001

Bibel M., Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev. 14(23):2919-37; 2000

Bibel M., Hoppe E., Barde YA. Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J. 18(3):616-22; 1999

Bonhoeffer T. Neurotrophins and activity-dependent development of the neocortex. Curr Opin Neurobiol. 6(1):119-26; 1996

Bresnahan PA., Leduc R., Thomas L., Thorner J., Gibson HL., Brake AJ., Barr PJ., Thomas G. Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-beta-NGF in vivo. J Cell Biol. 111(6 Pt 2):2851-9; 1990

Caleo M., Cenni MC. Anterograde transport of neurotrophic factors: possible therapeutic implications. Mol Neurobiol. 29(2):179-96; 2004

Campenot RB. NGF uptake and retrograde signaling mechanisms in sympathetic neurons in compartmented cultures. Results Probl Cell Differ. 48:141-58; 2009

Castrén E., Rantamäki T. Role of brain-derived neurotrophic factor in the aetiology of depression: implications for pharmacological treatment.CNS Drugs. 24(1):1-7; 2010

Catania S., Germanà A., Cabo R., Ochoa-Erena FJ., Guerrera MC., Hannestad J., Represa J., Vega JA. Neurotrophin and Trk neurotrophin receptors in the inner ear of Salmo salar and Salmo trutta. J Anat. 210(1):78-88; 2007

Ceccatelli S., Ernfors P., Villar MJ., Persson H., Hökfelt T. Expanded distribution of mRNA for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the rat brain after colchicine treatment. Proc Natl Acad Sci U S A. 88(22):10352-6; 1991

Chalazonitis A. Neurotrophin-3 as an essential signal for the developing nervous system. Mol Neurobiol. 12(1):39-53; 1996

Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci. 4(4):299-309; 2003

Chao MV. Retrograde transport redux. Neuron. 3;39(1):1-2; 2003

Chapouton P., Webb KJ., Stigloher C., Alunni A., Adolf B., Hesl B., Topp S., Kremmer E., Bally-Cuif L. Expression of hairy/enhancer of split genes in neural progenitors and neurogenesis domains of the adult zebrafish brain. J Comp Neurol. 15;519(9):1748-69; 2011

Chen QY., Chen Q., Feng GY., Wan CL., Lindpaintner K., Wang LJ., Chen ZX., Gao ZS., Tang JS., Li XW., He L. Association between the brain-derived neurotrophic factor (BDNF) gene and schizophrenia in the Chinese population. Neurosci Lett. 397(3):285-90; 2006

Cohen-Cory S., Lom B. Neurotrophic regulation of retinal ganglion cell synaptic connectivity: from axons and dendrites to synapses. Int J Dev Biol 48: 947–956; 2004

Conner JM., Lauterborn JC., Yan Q., Gall CM., Varon S. Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J Neurosci. 17(7):2295-313; 1997

Conner JM., Muir D., Varon S., Hagg T., Manthorpe M. The localization of nerve growth factor-like immunoreactivity in the adult rat basal forebrain and hippocampal formation. J Comp Neurol. 319(3):454-62; 1992

Conner JM., Varon S. Distribution of nerve growth factor-like immunoreactive neurons in the adult rat brain following colchicine treatment. J Comp Neurol. 326(3):347-62; 1992

Connor B., Dragunow M. The role of neuronal growth factors in neurodegenerative disorders of the human brain. Brain Res Brain Res Rev. 27(1):1-39; 1998

Covaceuszach S., Capsoni S., Cattaneo A. Development of a non invasive NGF-based therapy for Alzheimer's disease. Curr. Alzheimer Res. 6:158–170; 2009

Dalton VS., Borich SM., Murphy P., Roberts BL. Brain-derived neurotrophic factor mRNA expression in the brain of the teleost fish, *Anguilla anguilla*, the European Eel. Brain Behav Evol. 73(1):43-58; 2009a

Dalton VS., Roberts BL., Borich SM. Brain derived neurotrophic factor and trk B mRNA expression in the brain of a brain stem-spinal cord regenerating model, the European eel, after spinal cord injury. Neurosci Lett. 461(3):275-9; 2009b

D'Angelo L., de Girolamo P., Cellerino A., Tozzini ET., Castaldo L., Lucini C. Neurotrophin Trk receptors in the brain of a teleost fish, Nothobranchius furzeri. Microsc Res Tech.; 2011

Darnell DK., Stanislaw S., Kaur S., Antin PB. Whole mount in situ hybridization detection of mRNAs using short LNA containing DNA oligonucleotide probes. RNA 16(3):632-7; 2010

Davies AM. The role of neurotrophins in the developing nervous system. J Neurobiol. 25(11):1334-48; 1994

de Girolamo P., Arcamone N., Lucini C., Castaldo L., Vega JA., Gargiulo G. The teleost kidney expresses Trk neurotrophin receptor-like proteins. Anat Embryol (Berl) May;201(5):429-33; 2000

de Girolamo P., Lucini C., Vega JA., Andreozzi G., Coppola L., Castaldo L. Co-localization of Trk neurotrophin receptors and regulatory peptides in the endocrine cells of the teleostean stomach. Anat Rec. 256(3):219-26; 1999

Dethleffsen K., Heinrich G., Lauth M., Knapik EW., Meyer M. Insert-containing neurotrophins in teleost fish and their relationship to nerve growth factor. Mol Cell Neurosci. 24(2):380-94; 2003

Duprey-Díaz MV., Soto I., Blagburn JM., Blanco RE. Changes in brain-derived neurotrophic factor and trkB receptor in the adult Rana pipiens retina and optic tectum after optic nerve injury. J Comp Neurol. 454(4):456-69; 2002

Egan MF., Weinberger DR., Lu B. Schizophrenia, III: brain-derived neurotropic factor and genetic risk. Am J Psychiatry. 160(7):1242; 2003

Eide FF., Lowenstein DH., Reichardt LF. Neurotrophins and their receptors-current concepts and implications for neurologic disease. Exp Neurol. 121(2):200-14; 1993

Eisen JS., Pike SH., Romancier B. An identified motoneuron with variable fates in embryonic zebrafish. J Neurosci. 10(1):34-43; 1990

Ekström P., Ohlin LM. Ontogeny of GABA-immunoreactive neurons in the central nervous system in a teleost, gasterosteus aculeatus L. J Chem Neuroanat. 9(4):271-88; 1995

Ernfors P., Wetmore C., Olson L., Persson H. Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. Neuron. 5(4):511-26; 1990

Esposito D., Patel P., Stephens RM., Perez P., Chao MV., Kaplan DR., Hempstead BL. The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. J Biol Chem. 276(35):32687-95; 2001

Frade JM., Barde YA. Nerve growth factor: two receptors, multiple functions. Bioessays. 20(2):137-45; 1998

Friedman WJ., Black IB., Kaplan DR. Distribution of the neurotrophins brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 in the postnatal rat brain: an immunocytochemical study. Neuroscience 84(1):101-14; 1998

Friedman WJ., Ernfors P., Persson H. Transient and persistent expression of NT-3/HDNF mRNA in the rat brain during postnatal development J Neurosci. 11(6):1577-84; 1991

Ganz J., Kaslin J., Hochmann S., Freudenreich D., Brand M. Heterogeneity and Fgf dependence of adult neural progenitors in the zebrafish telencephalon. Glia 58(11):1345-63; 2010

Garofalo L., Ribeiro-da-Silva A., Cuello AC. Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals. Proc Natl Acad Sci U S A. 89(7):2639-43; 1992

Genade T., Benedetti M., Terzibasi E., Roncaglia P., Valenzano DR., Cattaneo A., Cellerino A. Annual fish of the genus Nothobranchius as a model system for aging research. Aging Cell 4(5): 223-33; 2005

Germanà A., González-Martínez T., Catania S., Laura R., Cobo J., Ciriaco E., Vega JA. Neurotrophin receptors in taste buds of adult zebrafish (Danio rerio) Neurosci Lett. 354(3):189-92; 2004

Germanà A., Laurà R., Montalbano G., Guerrera MC., Amato V., Zichichi R., Campo S., Ciriaco E., Vega JA. Expression of brain-derived neurotrophic factor and TrkB in the lateral line system of zebrafish during development. Cell Mol Neurobiol. 30(5):787-93; 2010a

Germanà A., Sánchez-Ramos C., Guerrera MC., Calavia MG., Navarro M., Zichichi R., García-Suárez O., Pérez-Piñera P., Vega JA. Expression and cell localization of brain-derived neurotrophic factor and TrkB during zebrafish retinal development. J Anat. 217(3):214-22; 2010b

Gillespie LN. Regulation of axonal growth and guidance by the neurotrophin family of neurotrophic factors. Clin Exp Pharmacol Physiol 30: 724–733; 2003

Götz R., Köster R., Winkler C., Raulf F., Lottspeich F., Schartl M., Thoenen H. Neurotrophin-6 is a new member of the nerve growth factor family. Nature 372(6503):266-9; 1994

Hallböök F., Ibáñez CF., Persson H. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron. May 6(5):845-58; 1991

Hallböök F., Lundin LG., Kullander K. Lampetra fluviatilis neurotrophin homolog, descendant of a neurotrophin ancestor, discloses the early molecular evolution of neurotrophins in the vertebrate subphylum. J Neurosci. 18(21):8700-11; 1999

Hallböök F., Wilson K., Thorndyke M., Olinski RP. Formation and evolution of the chordate neurotrophin and Trk receptor genes. Brain Behav Evol. 68(3):133-44; 2006

Hannestad J., Marino F., Germanà A., Catania S., Abbate F., Ciriaco E., Vega JA. Trk neurotrophin receptor-like proteins in the teleost Dicentrarchus labrax. Cell Tissue Res. 300(1):1-9; 2000

Hashimoto M., Heinrich G. Brain-derived neurotrophic factor gene expression in the developing zebrafish. Int J Dev Neurosci. 15(8):983-97; 1997

He XL., Garcia KC. Structure of nerve growth factor complexed with the shared neurotrophin receptor p75.Science. 304(5672):870-5; 2004

Hefti F. Neurotrophic factor therapy--keeping score. Nat Med. 3(5):497-8; 1997

Heinrich G., Lum T. Fish neurotrophins and Trk receptors. Int J Dev Neurosci. 18(1):1-27; 2000

Hempstead BL., Martin-Zanca D., Kaplan DR., Parada LF., Chao MV. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350, 678–683; 1991

Herzog KH., von Bartheld CS. Contributions of the optic tectum and the retina as sources of brain-derived neurotrophic factor for retinal ganglion cells in the chick embryo. J Neurosci. 18(8):2891-906; 1998

Hirokawa, N., Noda, Y., Tanaka, Y. & Niwa, S. Kinesinsuperfamily motor proteins and intracellular transport. *Nat. Rev. Mol. Cell Biol.* 10, 682–696; 2009

Hohn A., Leibrock J., Bailey K., Barde YA. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature. 344(6264):339-41; 1990

Huang EJ., Reichardt LF. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem. 72:609-42; 2003

Huber J. Killidata online http://www.killidata.org; 2005

Hyman C., Juhasz M., Jackson C., Wright P., Ip NY., Lindsay RM. Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. J Neurosci. 14(1):335-47; 1994

Ibáñez CF. Emerging themes in structural biology of neurotrophic factors. Trends Neurosci. 21(10):438-44; 1998

Ibáñez CF. Neurotrophic factors: from structure-function studies to designing effective therapeutics.Trends Biotechnol. 13(6):217-27;1995

Ip NY., Ibáñez CF., Nye SH., McClain J., Jones PF., Gies DR., Belluscio L., Le Beau MM., Espinosa R. 3rd, Squinto SP. Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci U S A. 89(7):3060-4; 1992

Ishikawa Y., Yoshimoto M., Ito H. A brain atlas of a wild-type inbred strain of the medaka, Oryzias latipes. The Fish Biology Journal MEDAKA, 10: 1-26; 1999

Ishikawa Y., Yoshimoto M., Yamamoto N., Ito H. Different brain morphologies from different genotypes in a single teleost species, the medaka (Oryzias latipes) Brain Behav Evol. 53(1):2-9; 1999

Ito H. A catalogue of histological preparations of the teleost brains. Med J Osaka Univ. 28(3-4):219-28; 1978

Jacobi S., Soriano J., Moses E. BDNF and NT-3 increase velocity of activity front propagation in unidimensional hippocampal cultures. J Neurophysiol. 104(6):2932-9; 2010

Jones KR., Reichardt LF. Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc Natl Acad Sci U S A. 87(20):8060-4; 1990

Jubb R. A new Nothobranchius (Pisces, Cyprinodontidae) from southeastern Rhodesia. J. Am. Killifish Assoc. 8: 12–19; 1972

Jubb R. Nothobranchius. Neptune City, New Jersey, T.F.H Publications; 1981

Kaisho Y., Yoshimura K., Nakahama K. Cloning and expression of a cDNA encoding a novel human neurotrophic factor. FEBS Lett. 266(1-2):187-91; 1990

Kamal A., Stokin GB., Yang Z., Xia CH., Goldstein LS. Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron. 28(2):449-59; 2000

Kamal A., Stokin GB., Yang Z., Xia CH., Goldstein LS. Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron. 28(2):449-59; 2000

Katoh-Semba R., Ichisaka S., Hata Y., Tsumoto T., Eguchi K., Miyazaki N., Matsuda M., Takeuchi IK., Kato K. NT-4 protein is localized in neuronal cells in the brain stem as well as the dorsal root ganglion of embryonic and adult rats. J Neurochem. 86(3):660-8; 2003

Kizawa-Ueda M., Ueda A., Kawamura N., Ishikawa T., Mutoh E., Fukuda Y., Shiroki R., Hoshinaga K., Ito S., Asakura K., Mutoh T. Neurotrophin levels in cerebrospinal fluid of adult patients with meningitis and encephalitis. Eur Neurol. 65(3):138-43; 2011

Kranz D., Richter W. Autoradiographic studies on the localization of the matrix zones of the diencephalon of young and adult Lebistes reticulatus (Teleostae). Z Mikrosk Anat Forsch.;82(1):42-66; 1970

Kubota K., Ohashi A., Imachi H., Harada H. Improved in situ hybridization efficiency with locked-nucleic-acid-incorporated DNA probes. Appl Environ Microbiol. 72(8):5311-7; 2006

Lai KO, Fu WY, Ip FC., Ip NY. Cloning and expression of a novel neurotrophin, NT-7, from carp. Mol Cell Neurosci. 11(1-2):64-76; 1998

Lanave C., Colangelo AM., Saccone C., Alberghina L. Molecular evolution of the neurotrophin family members and their Trk receptors. Gene. 394(1-2):1-12; 2007

Laramore C., Maymind E., Shifman MI. Expression of neurotrophin and its tropomyosin-related kinase receptors (Trks) during axonal regeneration following spinal cord injury in larval lamprey. Neuroscience 183:265-77; 2011

Lauterborn JC., Isackson PJ., Gall CM. Cellular localization of NGF and NT-3 mRNAs in postnatal rat forebrain. Mol Cell Neurosci. 5(1):46-62; 1994

Leibrock J., Lottspeich F., Hohn A., Hofer M., Hengerer B., Masiakowski P., Thoenen H., Barde YA. Molecular cloning and expression of brain-derived neurotrophic factor.Nature. 341(6238):149-52; 1989

Leingartner, A. and Lindholm, D., Two promoters direct transcription of the mouse NT-3 gene. Eur. J. Neurosci., 6(7), 1149-1159; 1994

Levi-Montalcini R. The nerve growth factor 35 years later. Science. 237(4819):1154-62; 1987

Levi-Montalcini R., Meyer H.,. Hamburger V. In vitro experiments on the effects of mouse sarcomas on the spinal and sympathetic ganglia of the chick embryo. Cancer Res. **14:** 49–57; 1954

Levi-Montalcini R., Skaper SD., Dal Toso R., Petrelli L., Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 19(11):514-20; 1996

Lewin GR, Barde YA. Physiology of the neurotrophins. Annu Rev Neurosci. 19:289-317; 1996

Li X., Franz J., Lottspeich F., Götz R. Recombinant fish neurotrophin-6 is a heparin-binding glycoprotein: implications for a role in axonal guidance. Biochem J. 324 (Pt 2):461-6; 1997

Liepinsh E., Ilag LL., Otting G., Ibáñez CF. NMR structure of the death domain of the p75 neurotrophin receptor. EMBO J. 16(16):4999-5005; 1997

Lindvall O., Ernfors P., Bengzon J., Kokaia Z., Smith ML., Siesjö BK., Persson H. Differential regulation of mRNAs for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the adult rat brain following cerebral ischemia and hypoglycemic coma. Proc Natl Acad Sci U S A. 89(2):648-52; 1992

Lisman JE., Harris KM. Quantal analysis and synaptic anatomy--integrating two views of hippocampal plasticity. Trends Neurosci. 16(4):141-7; 1993

Lucini C., de Girolamo P., Lamanna C., Botte V., Vega JA., Castaldo L. TrkA and TrkC neurotrophin receptor-like proteins in the lizard gut. Cell Tissue Res. 303(3):345-50; 2001

Lucini C., de Girolamo P., Maruccio L., Lamanna C., Castaldo L., Vega JA. Trk-neurotrophin receptor-like immunoreactivity in the gut of teleost species. Cell Tissue Res. 296(2):323-30; 1999

Lucini C., Maruccio L., de Girolamo P., Vega JA., Castaldo L. Localisation of neurotrophin - containing cells in higher vertebrate intestine. Anat Embryol (Berl) 205(2):135-40; 2002

Lyons WE., Mamounas LA., Ricaurte GA., Coppola V., Reid SW., Bora SH., Wihler C., Koliatsos VE., Tessarollo L. Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. Proc Natl Acad Sci U S A. 96(26):15239-44; 1999

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247(4949 Pt 1):1446-51; 1990

Maisonpierre PC., Belluscio L., Friedman B., Alderson RF., Wiegand SJ., Furth ME., Lindsay RM., Yancopoulos GD. NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron. 5(4):501-9; 1990

Maisonpierre PC., Le Beau MM., Espinosa R., Ip NY., Belluscio L., de la Monte SM., Squinto S., Furth ME., Yancopoulos GD.Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations.Genomics. 10(3):558-68; 1991

Maness LM., Kastin AJ., Webber JT., Banks WA., Beckman BS., Zadina JE. The neurotrophins and their receptors: structure, function, and neuropathology, Neurosci. Biobehav. Rev. 18(1):143-59; 1994

Manger PR., Cort J., Ebrahim N., Goodman A., Henning J., Karolia M., Rodrigues SL., Strkalj G. Is 21st century neuroscience too focussed on the rat/mouse model of brain function and dysfunction? Front Neuroanat 2:5; 2008

Martin-Zanca D., Oskam R., Mitra G., Copeland T., Barbacid M. Molecular and biochemical characterization of the human trk proto-oncogene. Mol Cell Biol.; 9(1):24-33; 1989

McDonald NQ., Hendrickson WA. A structural superfamily of growth factors containing a cystine knot motif.Cell. 73(3):421-4; 1993

McTigue PM., Peterson RJ., Kahn JD. Sequence-dependent thermodynamic parameters for locked nucleic acid (LNA)-DNA duplex formation. Biochemistry 43(18):5388-405; 2004

Meyer RL. Evidence from thymidine labeling for continuing growth of retina and tectum in juvenile goldfish. Exp Neurol. 59(1):99-111; 1978

Mitra G., Martin-Zanca D., Barbacid M. Identification and biochemical characterization of p70TRK, product of the human TRK oncogene. Proc Natl Acad Sci U S A. 84(19):6707-11; 1987

Mueller T., Vernier P., Wullimann MF. The adult central nervous cholinergic system of a neurogenetic model animal, the zebrafish Danio rerio. Brain Res. 1011(2):156-69; 2004

Nagahara AH., Merrill DA., Coppola G., Tsukada S., Schroeder BE., Shaked GM., Wang L., Blesch A., Kim A., Conner JM., Rockenstein E., Chao MV., Koo EH., Geschwind D., Masliah E., Chiba AA., Tuszynski MH. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. Nat Med. 15(3):331-7; 2009

Nieuwenhuys R. The forebrain of actinopterygians revisited. Brain Behav Evol. 73(4):229-52; 2009

Nieuwenhuys R., ten Donkelaar HJ., Nicholson C., Smeets WJAJ., Wicht H., Meek J., Dubbeldam JL., van Dongen PAM., Voogd J. The Central Nervous System of Vertebrates. Springer; 1 edition (Nov 25 1997)

Niewiadomska G., Mietelska-Porowska A., Mazurkiewicz M. The cholinergic system, nerve growth factor and the cytoskeleton. Behav Brain Res. 221(2):515-26; 2011

Nilsson AS., Fainzilber M., Falck P., Ibáñez CF. Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. FEBS Lett. 424(3):285-90; 1998

Northcutt R G. Connections of the lateral and medial divisions of the goldfish telencephalic pallium. *Journal of Comparative Neurology* 494, 903–943; 2006

Papaliagkas VT., Tsolaki M., Anogianakis G. Cerebrospinal fluid proteins in the diagnosis of Alzheimer's disease. Recent Pat CNS Drug Discov. 5(2):172-8; 2010

Pellegrini E., Mouriec K., Anglade I., Menuet A., Le Page Y., Gueguen MM., Marmignon MH., Brion F., Pakdel F., Kah O. Identification of aromatase-positive radial glial cells as progenitor cells in the ventricular layer of the forebrain in zebrafish. J Comp Neurol. 501(1):150-67; 2007

Peter RE., Macey MJ., Gill VE. A stereotaxic atlas and technique for forebrain nuclei of the killfish, Fundulus heteroclitus. J Comp Neurol. 159(1):103-27; 1975

Petersen M. and Wengel J. LNA: a versatile tool for therapeutics and genomics. Trends Biotechnol. 21, 74-81; 2003

Pierce JP., Lewin GR. An ultrastructural size principle. Neuroscience. 58(3):441-6; 1994

Pike SH., Melancon EF., Eisen JS. Pathfinding by zebrafish motoneurons in the absence of normal pioneer axons. Development. 114(4):825-31; 1992

Prakash N., Cohen-Cory S., Frostig RD. RAPID and opposite effects of BDNF and NGF on the functional organization of the adult cortex in vivo. Nature. 381(6584):702-6; 1996

Purves D. Body and Brain, A Trophic Theory of Neural Connections, Harvard University Press, Cambridge, MA; 1988

Quartu M., Serra MP., Boi M., Melis T., Ambu R., Del Fiacco M. Brain-derived neurotrophic factor (BDNF) and polysialylated-neural cell adhesion molecule (PSA-NCAM): codistribution in the human brainstem precerebellar nuclei from prenatal to adult age. Brain Res. 1363:49-62; 2010

Raymond PA., Easter SS Jr. Postembryonic growth of the optic tectum in goldfish. I. Location of germinal cells and numbers of neurons produced. J Neurosci. 3(5):1077-91; 1983

Reichard M., Polačik M., Sedláček O. Distribution, colour polymorphism and habitat use of the African killifish, *Nothobranchius furzeri*, the vertebrate with the shortest lifespan. J Fish Biol. 74(1): 198-212; 2009

Reichardt LF. Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci. 361(1473):1545-64; 2006

Riddle DR., Lo DC., Katz LC. NT-4-mediated rescue of lateral geniculate neurons from effects of monocular deprivation. Nature. 9;378(6553):189-91; 1995

Rink E., Wullimann MF. Connections of the ventral telencephalon and tyrosine hydroxylase distribution in the zebrafish brain (Danio rerio) lead to identification of an ascending dopaminergic system in a teleost. Brain Res Bull. 57(3-4):385-7; 2002

Rink E., Wullimann MF. The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). Brain Res. 889(1-2):316-30; 2001

Robinson RC., Radziejewski C., Stuart DI., Jones EY. Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer. Biochemistry. 34(13):4139-46; 1995

Rocamora, N., García-Ladona, F.J., Palacios, J.M., Mengod, G. Differential expression of brain-derived neurotrophic factor, neurotrophin-3, and low-affinity nerve growth factor receptor during the postnatal development of the rat cerebellar system. Mol. Brain Res. 17, 1–8; 1993

Rodríguez-Tébar A., Dechant G., Barde YA. Neurotrophins: structural relatedness and receptor interactions.Philos Trans R Soc Lond B Biol Sci. 331(1261):255-8; 1991

Rosenthal A., Goeddel DV., Nguyen T., Lewis M., Shih A., Laramee GR., Nikolics K., Winslow JW. Primary structure and biological activity of a novel human neurotrophic factor. Neuron.;4(5):767-73; 1990

Rothenaigner I., Krecsmarik M., Hayes JA., Bahn B., Lepier A., Fortin G., Götz M., Jagasia R., Bally-Cuif L. Clonal analysis by distinct viral vectors identifies bona fide neural stem cells in the adult zebrafish telencephalon and characterizes their division properties and fate. Development 138(8):1459-69; 2011

Ruit KG., Osborne PA., Schmidt RE., Johnson EM. Jr., Snider WD. Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. J Neurosci. 10(7):2412-9; 1990

Russo SJ., Mazei-Robison MS., Ables JL., Nestler EJ. Neurotrophic factors and structural plasticity in addiction. Neuropharmacology. 1:73-82; 2009

Schecterson LC., Bothwell M. Neurotrophin receptors: Old friends with new partners. Dev Neurobiol. 70(5):332-8; 2010

Schliebs R., Arendt T. The cholinergic system in aging and neuronal degeneration. Behav Brain Res. 221(2):555-63; 2011

Schnell L., Schneider R., Kolbeck R., Barde YA., Schwab ME. Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature. 367(6459):170-3; 1994

Seegers L. Old World Killis II. Germany: Mörfelden-Walldorf. , Aqualog; 1997

Seidah NG., Benjannet S., Pareek S., Chrétien M., Murphy RA. Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS Lett. 379(3):247-50; 1996 (a)

Seidah NG., Benjannet S., Pareek S., Savaria D., Hamelin J., Goulet B., Laliberte J., Lazure C., Chrétien M., Murphy RA. Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases.Biochem J. 314 (Pt 3):951-60; 1996 (b)

Setiamarga DH., Miya M., Yamanoue Y., Mabuchi K., Satoh TP., Inoue JG., Nishida M. Interrelationships of Atherinomorpha (medakas, flyingfishes, killifishes, silversides, and their relatives): The first evidence based on whole mitogenome sequences. Mol Phylogenet Evol. 49(2):598-605; 2008

Shantanu K. and Deepak B. Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino. Appl Microbiol Biotechnol 71: 575–586; 2006

Shelton DL., Reichardt LF. Studies on the expression of the beta nerve growth factor (NGF) gene in the central nervous system: level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. Proc Natl Acad Sci U S A. 83(8):2714-8; 1986

Stanzione F., Esposito L., Paladino A., Pedone C., Morelli G., Vitagliano L. Role of the conformational versatility of the neurotrophin N-terminal regions in their recognition by Trk receptors. Biophys J. 99(7):2273-8; 2010

Sun PD., Davies DR. The cystine-knot growth-factor superfamily. Annu Rev Biophys Biomol Struct. 24:269-91; 1995

Talamini LM., Aloe L. Immunohistochemical localization of nerve growth factor (NGF) and NGF-receptor in the hypothalamus of adult rats. Arch Ital Biol. 131(4):255-66; 1993

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 28(10):2731-9; 2011

Terzibasi E., Valenzano DR., Cellerino A. The short-lived fish Nothobranchius furzeri as a new model system for aging studies. Exp Gerontol. 42(1-2):81-9; 2007

Tettamanti G., Cattaneo AG., Gornati R., de Eguileor M., Bernardini G., Binelli G. Phylogenesis of brain-derived neurotrophic factor (BDNF) in vertebrates. Gene. 450(1-2):85-93; 2010

Thoenen H. Neurotrophins and neuronal plasticity. Science. 270(5236):593-8; 1995

Timmusk T., Belluardo N., Metsis M., Persson H. Widespread and developmentally regulated expression of neurotrophin-4 mRNA in rat brain and peripheral tissues. Eur J Neurosci. 5(6):605-1; 1993

Tongiorgi E. Activity-dependent expression of brain-derived neurotrophic factor in dendrites: facts and open questions. Neurosci Res. 61(4):335-46; 2008

Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 35 (Web Server issue):W71-4; 2007

Vaegter CB., Jansen P., Fjorback AW., Glerup S., Skeldal S., Kjolby M., Richner M., Erdmann B., Nyengaard JR., Tessarollo L., Lewin GR., Willnow TE., Chao MV., Nykjaer A. Sortilin associates with Trk receptors to enhance anterograde transport and neurotrophin signaling. Nat Neurosci. 14(1):54-61. 2010

Valdesalici S., Cellerino A. Extremely short lifespan in the annual fish Nothobranchius furzeri. Proc Biol Sci. 270 Suppl 2:S189-91; 2003

van Kesteren RE., Fainzilber M., Hauser G., van Minnen J., Vreugdenhil E., Smit AB., Ibáñez CF., Geraerts WP., Bulloch AG. Early evolutionary origin of the neurotrophin receptor family. EMBO J. 17(9):2534-42; 1998

Vígh B., Manzano e Silva MJ., Frank CL., Vincze C., Czirok SJ., Szabó A., Lukáts A., Szél A. The system of cerebrospinal fluid-contacting neurons. Its supposed role in the nonsynaptic signal transmission of the brain. Histol Histopathol. 19(2):607-28; 2004

Vissio PG., Cánepa MM., Maggese MC. Brain-derived neurotrophic factor (BDNF)-like immunoreactivity localization in the retina and brain of *Cichlasoma dimerus* (Teleostei, Perciformes). Tissue Cell. 40(4):261-70; 2008

von Bartheld CS. Neurotrophins in the developing and regenerating visual system. Histol Histopathol. 13(2):437-59; 1998

Wahlestedt C., Salmi P., Good L., Kela J., Johnsson T., Hökfelt T., Broberger C., Porreca F., Lai J., Ren K., Ossipov M., Koshkin A., Jakobsen N., Skouv J., Oerum H., Jacobsen MH., Wengel J. Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. Proc Natl Acad Sci U S A 97(10):5633-8; 2000

Wengel J. Synthesis of 30-C- and40-C-branched oligonucleotides and the development of locked nucleic acid (LNA). Acc. Chem. Res. 32, 301–310; 1998

Wildekamp RH. A World of Killies: Atlas of the Oviparous Cyprinodontiform Fishes of the World, IV. New York, USA: American Killifish Association; 2004

Wirth MJ., Patz S., Wahle P. Transcellular induction of neuropeptide Y expression by NT4 and BDNF. Proc Natl Acad Sci U S A. 102(8):3064-9; 2005

Woo SB., Whalen C., Neet KE. Characterization of the recombinant extracellular domain of the neurotrophin receptor TrkA and its interaction with nerve growth factor (NGF). Protein Sci. 7(4):1006-16; 1998

Wourms JP. Developmental biology of annual fishes. I. Stages in the normal development of Austrofundulus myersi Dahl. J Exp Zool 182(2): 143-67; 1972a

Wourms JP. The developmental biology of annual fishes. II. Naturally occurring dispersion and reaggregation of blastomers during the development of annual fish eggs. J Exp Zool 182(2): 169-200; 1972b

Wourms JP. The developmental biology of annual fishes. III. Preembryonic and embryonic diapause of variable duration in the eggs of annual fishes. J Exp Zool 182(3): 389-414; 1972c

Wullimann MF., Mueller T. Teleostean and mammalian forebrains contrasted: evidence from genes to behavior. J Comp Neurol. 475(2):143-62; 2004

Yang CY., Yoshimoto M., Xue HG., Yamamoto N., Imura K., Sawai N., Ishikawa Y., Ito H. Fiber connections of the lateral valvular nucleus in a percomorph teleost, tilapia (Oreochromis niloticus). J Comp Neurol. 474(2):209-26; 2004

Zafra F., Hengerer B., Leibrock J., Thoenen H., Lindholm D. Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J. 9(11):3545-50; 1990

Zhou XF., Rush RA. Localization of neurotrophin-3-like immunoreactivity in the rat central nervous system. Brain Res. 643(1-2):162-72; 1994

Zikopoulos B., Kentouri M., Dermon CR. Proliferation zones in the adult brain of a sequential hermaphrodite teleost species (Sparus aurata). Brain Behav Evol. 56(6):310-22; 2000

Zupanc GK., Hinsch K., Gage FH. Proliferation, migration, neuronal differentiation, and long-term survival of new cells in the adult zebrafish brain. J Comp Neurol.;488(3):290-319; 2005

Zupanc GK., Horschke I. Proliferation zones in the brain of adult gymnotiform fish: a quantitative mapping study. J Comp Neurol. 353(2):213-33; 1995

Zupanc GK., Horschke I., Ott R., Rascher GB. Postembryonic development of the cerebellum in gymnotiform fish. J Comp Neurol. 370(4):443-64; 1996

Zupanc GKH. and Sîrbulescu RF. Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish European Journal of Neuroscience, Vol. 34, pp. 917–929, 2011

9. ACKNOWLEDGMENTS

I am truly thankful to many people, that have helped and supported me to design, implement and carry out the work described in this thesis. There is no way to acknowledge them all, or even any of them properly. I sincerely hope that everyone who knows that they have influenced me feels satisfaction that they have helped me.

First, I want to thank my advisor Prof Paolo de GIROLAMO. It has been a honor to be his first PhD student. He has taught me, consciously and unconsciously, how good research work is done. I appreciate all his contributions of time, ideas, and funding to make my PhD experience productive and stimulating. The joy and enthusiasm he has for the research was contagious and motivational for me, even during tough times in the PhD pursuit. I am also thankful for the excellent example he has provided as person and professor.

I'm grateful to Prof Carla LUCINI. I have truly appreciated the amount of time and energy she has invested in guiding me throughout this work, her support in the most critical moments, the many enriching scientific discussions. She has been for me a mentor and good companion.

I want to express my special gratitude to Prof Luciana CASTALDO for her continuing support and encouragement, her enthusiasm in sharing her broad knowledge, and the many conversations, as enlightening as always, and her patience for the editing work of the thesis.

This Thesis owes a lot to Dr Alessandro CELLERINO and Dr Eva TERZIBASI TOZZINI, who have offered me the opportunity of going to FLI-Leibniz Institute of Jena (Germany), to develop the biomolecular part of this research project. They have provided me the indispensable ingredients for realizing a more complete study on the model organism and the molecular biology aspects of the thesis. I'm also grateful to all members of the Cellerino's Group of FLI-Leibniz Institute of Jena (Germany), for their help and invaluable contribution to this work.

I’m also thankful to Prof Alessandro FIORETTI and Prof Lucia Francesca MENNA, for the lively discussions and the formative influence they had on my adult life.

A special thank to Antonio CALAMO, who has generously shared his time and his graphic competences with me.

Moreover, I would like to extend my gratitude to Michelino, Sabrina and all people who contribute in making the Department of Biological Structures, Functions and Technologies such a stimulating, lively and friendly place.

My time at the Department was made enjoyable in large part due to the friends/colleagues: Bernadette, Claudia and Bruna. Thanks for having shared time, complaints and confidences with me. And for the coffee!

Particular gratitude goes to my friends, for being by my side when I needed, for supporting me, for encouraging me, for distracting me joyously in the tough times of the PhD period.

Finally, I owe special acknowlegments to my family for continuous and unconditional support. I dedicate this thesis to my mother, my father, Floriana and Giangiacomo.